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Donor-specific cell mediated lympholysis responsiveness and suppressor cell studies in renal allograft recipients

by

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A THESIS

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ABSTRACT

Donor-specific immunological adaptive mechanisms are believed to be important in the long term functioning of HLA mismatched grafts. To assess this phenomenon, the immunological responsiveness of a panel of 30 renal allograft recipients (R), was studied against the cells of the specific donor (D). Fifteen recipients had rejected their transplants (Tx) within one year, while the other 15 had good function at the same period.

Serial samples were obtained from all the patients, pre Tx (R_0), post Tx zero to thirty days (R_1), thirty-one to ninety days (R_2) and ninety-one days to one year (R_3). Two donor specific immunological systems were used. In the first, the cell mediated lympholysis (CML) inducibility of pre and post Tx R cells to stimulation by irradiated D or healthy controls (Y) were compared (R_0Dx, R_1Dx, etc.). In the second, the suppressive capacity of irradiated post Tx R lymphocytes on CML inducibility was assayed ($R_0Dx + R_1x$, etc.). These two assays were run simultaneously on all the samples in individual patients.

In the fifteen patients with rejected allografts, the mean CML specific lysis of R_0Dx was 47% post Tx R_1Dx remained elevated at 37%, and R_2Dx at 41%. Control values, R_0Yx, R_1Yx and R_2Yx were 55, 52 and 50%, respectively. Suppressor cell studies in this group showed no significant difference between patients and controls, i.e., no

donor-specific suppression. In the other 15 patients with good function R.Dx was 40%, all the post Tx samples dropped to 20% or less ($P<0.005$) with no drops in the controls. Suppressor cell studies in this group showed a small but statistically significant suppression ($p<0.02$). Further characterization of the suppressor cell population suggested that they were radiosensitive and belonged to the T suppressor/cytotoxic enriched population. Kinetic studies performed with the CML assay showed no evidence of accelerated kinetics.

These results suggest that the CML inducibility post Tx may be useful as a predictive index of ultimate transplant outcome.

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ABBREVIATIONS USED

1. ABS - human AB serum
2. ADCC - antibody dependent cell-mediated cytotoxicity
3. AET - aminoethylisothiouronium hydrobromide
4. CDC - complement dependent cytotoxicity
5. Con A - concanavalin A
6. CML - cell mediated lympholysis
7. DR - D region related
8. DMSO - dimethyl sulfoxide
9. E-RFC - erythrocyte - rosette forming cell
10. FCS - fetal calf serum
11. F-H - ficoll hypaque
12. H-2 - major histocompatibility complex of mouse
13. HLA - human leukocyte antigens
14. Ia - I region associated
15. LD - lymphocyte defined
16. LMC - lymphocyte mediated cytotoxicity
17. MHC - major histocompatibility complex
18. MLC - mixed lymphocyte culture
19. PBL - peripheral blood lymphocytes
20. PHA - phytohemagglutinin
21. PHS - pooled human serum
22. SRBC - sheep red blood cells
23. S.C. - suppressor cell
24. S.G. - specific gravity
25. T.h. - T helper lymphocytes
26. T.s/c. - T suppressor/cytotoxic lymphocytes

27. UV - ultraviolet

I. INTRODUCTION AND LITERATURE REVIEW

A. Introduction

Twenty six years have elapsed since the first successful renal transplant between non-identical twins. Early in 1959, John Merrill and his group performed a renal transplant between non-identical twins after irradiating the recipient (Merrill et al., 1960), the allograft survived more than four years. A few months later, Hamburger's group at the Necker Hospital, performed a similar procedure (Hamburger et al., 1962). Since those initial successes, over 80,000 renal transplants have been performed worldwide. This dramatic explosion has resulted primarily from two major developments in the transplantation field: (1) the development of effective immunosuppressive drugs and (2) the discovery of the HLA system.

The importance of HLA matching for living related donor transplants has been well documented, but there is some controversy on its value in cadaver donor allografts. However, with longer follow-up periods and matching for the DR antigen, its importance in cadaver allograft survival has been accepted (Moen et al., 1982).

Over the last two decades a sizable number of long-term survivors have accrued. A large majority of these recipients have normally functioning allografts despite a HLA nonidentity between the donor and recipient in most cases. The questions raised by these observations are: why are

these non-identical grafts not rejected? What are the mechanisms involved in the production of this state of apparent tolerance?

The purpose of this study was to assess the importance of donor-specific cell mediated responses in the survival of an allograft, and in individuals who have achieved a certain degree of tolerance to the allograft, to define the role of suppressor cells in the production of this phenomenon.

The hypothesis at the outset of the study was that cell mediated cytotoxicity as detected by the cell mediated lympholysis assay does show a correlation with the function of the allograft, and in addition suppressor cells are an important part of the mechanism of tolerance to allografts.

The primary assay used in this study is the mixed lymphocyte reaction, specifically assaying the function of cytotoxic lymphocytes in this reaction. This in vitro model of antiallogeneic reaction appears to correlate with the sequence of events following an in vivo allograft.

B. Mixed lymphocyte culture (MLC)

The study of MLC with lymphocyte populations of two different individuals in the same test tube was first suggested in 1964 by Fritz Bach in the United States (Bach et al., 1966) and Barbara Bain in Canada (Bain et al., 1964). They showed that contact of one population with the other triggers a profound transformation of the lymphocytes.

In order to study separately the reaction of one population against the other, one of the lymphocyte populations was inhibited by irradiation or treatment with mitomycin. This procedure was termed the one way MLC.

The first stage of the reaction is the recognition of histocompatibility antigens of the stimulator cells by the responding cells. These responding cells are T lymphocytes, as demonstrated by the absence of any MLC if lymphocytes are taken from T cell deprived animals, for example, animals thymectomized at birth. The presence of cells from the monocyte-macrophage series in the culture is also necessary for this initial step of allogeneic recognition.

The second stage of the reaction is the transformation and proliferation of the stimulated lymphocytes. The transformation is often termed blastogenesis, since the cells are similar to immature lymphoblasts. These transformed blasts are large cells with abundant basophilic cytoplasm and a nucleus with increased synthetic activity measured by the incorporation of tritiated thymidine.

The other main element of the mixed lymphocyte reaction is the production of cytotoxic lymphocytes, whose cytotoxicity is specifically directed against cells bearing histocompatibility antigens of the stimulator cells. These cytotoxic properties of the lymphocytes are evaluated by the cell mediated lympholysis test (CML).

The sequence of the MLC described is under the control of both regulatory cells as well as soluble mediators termed

lymphokines. Some cells can enhance the reaction, the T cell subset described as T helper cells may considerably increase the development of cytotoxic cells. Suppressor T cells may inhibit the reaction, and can be found in the initial few days of the MLC.

Hence, the MLC appears to be a complex process involving various types of cell subsets and soluble factors, some of which enhance and others depress the reaction. The final result depends on the balance between opposite forces. This image is quite applicable to the in vivo situation of a renal allograft where the balance between rejection and antirejection forces decides whether the allograft is destroyed or tolerated.

C. In vivo Correlation of Mixed Leucocyte Reaction and Cytotoxicity Assay

The extent to which the above phenomena described in in-vitro models actually occur in vivo in the allograft recipient has been studied. Here too, a series of stages can be defined, recognition of the graft antigens, stimulation of the host immunocompetent cells, production and proliferation of cytotoxic lymphocytes.

Alloantigen recognition is thought to involve primarily the T lymphocytes of the recipient and the histocompatibility antigens of the donor. The poor allograft response of animals thymectomized at birth supports the importance of T cells in this response.

The recognition stage is believed to occur locally in the graft and implies close contact between the host and allogeneic cells. The phenomenon of spontaneous rosettes suggests that contact accompanying recognition does occur; for example, lymphocytes from species A when mixed with RBC's from species B will form spontaneous rosettes, i.e., without preimmunization, and depleting a spleen population of rosette forming cells to a specific antigen, induces unresponsiveness to that antigen (Bach, 1970).

Blast transformation takes place in vivo in allografted animals. Evidence for this has been produced in kidney allografts in sheep (Pedersen and Morris, 1970). Lymphocytes leaving an allografted kidney can be collected by catheterizing the renal lymphatics, during rejection the normal cells have been transformed into numerous blast cells. This finding has been confirmed in human renal allografts (Hamburger et al., 1971).

Cytotoxic cells directed against cells of the specific donor have been demonstrated. In rats following renal allografts donor-specific cytotoxic lymphocytes can be found in the lymph nodes and the spleen (Biesecker, 1973).

In human renal allografts undergoing rejection, infiltrating cells in the graft show specific cytotoxicity (Strom et al., 1975). In addition, in vivo generated cytotoxic cells against the donor cell target are frequently detected in the lymphocyte mediated cytotoxicity (LMC) test (Dossetor et al., 1978). These correlations would suggest

that cytotoxic lymphocytes are an important element in cellular rejection.

Macrophages probably have multiple roles in the allogeneic reaction. They probably facilitate antigen recognition by T lymphocytes, they acquire a nonspecific cytotoxicity under the effect of lymphokines, such as macrophage activation factor, released by sensitized lymphocytes; and finally they may control, to some extent, the intensity of the lymphocyte reaction (North et al., 1978).

The important role of macrophages in vivo is suggested by the high percentage of host macrophages invading allografts during rejection (Dy et al., 1979).

In short, the in vitro assay of the generation of cytotoxic T cells in mixed leukocyte culture (MLC-induced CML) does seem to show a good correlation with cellular mechanisms operating in vivo following allografting.

D. Induction of Specific Tolerance to Allografts

Most transplant centres have some patients who have stopped immunosuppression for various reasons, but still retain a functioning allograft. Murray et al. (1964) found that some dogs did not reject a kidney after immunosuppression was stopped. One of the initial renal transplants in man has tolerated his kidney for over twenty years after the initial total body radiation (Bach et al., 1981). However, results of this sort are uncommon and

difficult to predict. There have been numerous studies, and our knowledge in this area has increased significantly, but it has also given rise to some of the most hotly debated fundamental questions in the whole of transplantation immunology. There is still no practical method available for inducing specific tolerance to an allografted organ, nevertheless there is a strong likelihood that the final solution to the problem of organ transplantation will lie in the creation of a state of specific tolerance.

E. Immunological Tolerance

Tolerance is a specific lack of reactivity to an antigen despite prior exposure to the same antigen. There are various factors which influence development of tolerance to an antigen, these include the dose, nature and persistence of the antigen, the route of immunization, as well as the age of the individual.

Some of the initial experiments that led to the concept of immunological tolerance were performed by Medawar and Billingham (1956). They showed that administration of an antigen immediately before or soon after birth renders the animal incapable of subsequent response to that antigen. Prior to this, Owen in 1945 first noted transplantation tolerance by way of blood group chimerism in dizygotic cattle twins. Each twin contained permanently its own blood type and that of its twin. Later, it was shown that kidney grafts from the dizygotic twin were tolerated by eight out

of twelve such animals for more than ten months (Craigle et al., 1968).

Burnett and Fenner (1949) developed the idea that recognition of self is acquired in embryonic life and further exploration of this idea led Burnett to postulate his famous clonal selection hypothesis.

Triplett (1962) took the pituitary from an embryonic tree frog, parked it for 10 days in another embryo, and then returned it to the donor. The original donor turned black in minutes due to the release of melanophore stimulating hormone and then albino again as the graft was rejected in 20 to 60 days. When the same experiment was done with half a pituitary, no rejection occurred. Rejection in the first instance, he felt, was due to deprivation of the tissue antigen. This led to the concept that tolerance had to be learned and the learning process required the presence of antigen.

F. Development of Allograft Tolerance

Cultured allografts of both thyroid and pancreatic islet tissue carry recognizable antigen and are promptly rejected if recipient animals are challenged with donor leukocytes at the time of transplantation. However, animals carrying allografts for prolonged periods (>100 days) become progressively more resistant to challenge with donor cells (Bowen et al., 1981). This phenomenon might develop as the result of the slow leakage of free antigen into the immune

system of the recipient, leading to tolerance induction.

In pancreatic islet transplantation, it was observed that when transplanted animals are injected with uv-irradiated donor spleen cells, around thirty days post-transplantation, graft rejection is not stimulated; administration of viable donor cells at this time promptly activate a rejection reaction. When animals that received uv irradiated cells were subsequently challenged with viable donor spleen cells, the allografts were not rejected. The uv irradiated cells, which provide a source of antigen alone, have stimulated the development of tolerance in the allografted animal.

G. Mechanisms of Immune Tolerance

There are several postulated mechanisms in the production of allograft tolerance. The primary ones are enhancement, antiidiotypic immunization, suppression by T cells and clonal deletion.

H. Enhancement

There have been many reports of considerable prolongation of kidney allograft survival in experimental animals by passive or active enhancement. Monaco (1966) showed that the rat is particularly suitable for successful enhancement of kidney grafts. However, dogs in enhancement experiments show prolonged survival only with the addition of small doses of azathioprine or prednisolone (Wilson et

al., 1971). The mechanism and site of action of enhancing antibodies is not definite. They could intervene by preventing recipient sensitization or by opposing the action of cytotoxic T cells. In addition, they could act on regulatory mechanisms by stimulating suppressor cells (Voisin et al., 1980), or by antiidiotypic antibody (Binz et al., 1979).

I. Antiidiotypic Immunization

The antigen binding sites of antibodies express antigens, the idiotypes, that are characteristic of the antibody specificity. The injection into an animal of antiidiotypic antibodies will suppress, if adequate protocols are used, the production of antibodies showing the corresponding idiotypes (Cosenza, 1972). The demonstration of the presence of common idiotypes on both T and B cells receptors suggests that these antibodies could also act at the T cell level and inhibit cell mediated immunity.

Binz and Wigzell (1979) demonstrated that they could induce a certain degree of transplantation tolerance in the rat model using the principle of antiidiotypic immunization. They inoculated T cell molecules of the specificity Lewis anti-DA, which were isolated from normal Lewis serum, into normal Lewis rats. The response in the MLC of such autoimmunized animals against DA stimulator cells was drastically reduced, while the response against the third party alloantigens was unaffected. In addition, other T cell

functions such as CML and graft vs host (GVH) reaction were also shown to be inhibited specifically. Autoimmunized Lewis rats that were shown to be specifically suppressed in the MLC were tested for their ability to reject DA skin grafts. The survival time in these rats was prolonged to two-and-one-half times the control time.

More recently, Suciu-Foca (1983) and her group have shown a role of antiidiotypic antibodies in down-regulation of alloimmunity, following donor-specific transfusions (DST) and transplantation. Their experiments suggest that allosensitization occurs in all patients who receive DST and transplants, but responsiveness or non-responsiveness are merely a reflection of the idiotype-antiidiotype antibody ratio (Reed et al., 1985).

J. Clonal Deletion

The original notion of the mechanism of tolerance was clonal deletion. It was thought that antigen reacted with a lymphocyte surface receptor, and because of the form of the antigen or the state of the lymphocyte, either induction of an immune response or induction of tolerance occurred. It is not known if specific antigen reactive clones of cells are actually deleted in tolerance or if they are present whether they are functionally unable to respond to the immunogenic antigen.

Evidence supporting the view that functional clonal deletion does occur among antiallogeneic T lymphocytes was

provided by Nossal and Pike (1981). To estimate the number of T cells active against a given antigen, they enumerated the frequencies of precursors of cytotoxic T lymphocytes (CTL-P) which developed from single cells, by appropriate stimulations with irradiated allogeneic cells acting as the antigen source. They applied this method to the classical tolerance model of newborn mice receiving semiallogeneic spleen cells.

CBA (H-2k) mice were rendered tolerant to H-2d antigens by injection of (CBA x BALB/c) F_1 spleen cells on the day of birth. At intervals of two days to twelve weeks, the frequencies of anti-H-2d and CTL-P in the thymus and spleen were determined by limiting dilution technique. A profound and long lasting functional clonal deficit was noted. The functional clonal deletion reduced the observed proportion of anti-H-2d cells in adult spleens from a normal level of about 1 in 500 spleen cells (1 in 150 T cells) to 3% of that figure. Evidence to support this functional clonal deletion has been provided by Streilein's group who failed to detect lymphocytes reactive with tolerated H-2 alloantigens in a wide battery of tests including MLC, CML, GVH reactions. Suppressor cells were not found in these studies, and the inference was of an active process achieving specific clonal deletion (Gruchalla et al., 1982).

To assess whether suppressor cells could be responsible for this functional clonal deletion, Nossal and Pike investigated the effects of anti-IJ serum on tolerance

induction in their model. They found that repeated high doses of anti-IJ injected into newborns partially inhibited clonal deletion. Though the phenomenon was reproducible, it depended on a particular batch of anti-IJ serum, which so far has not been reproduced with other batches (Nossal, 1983). The role of suppressor cells in functional clonal deletion therefore remains obscure.

In human renal transplantation, Pfeffer and Thorsby (1983) reached the conclusion that the weak donor-specific cytotoxic response is caused by a depletion of cytotoxic clones with high lytic efficiency. They could not find evidence for suppressor cells in the majority of their patients with reduced CML, and also found that addition of exogenous interleukin-2 (IL-2) did not improve cytotoxicity against the donor.

K. Suppressor Cells

History

The initial concept of suppressor cells was introduced by Gershon and Kondo in 1970. They found that lymphoid cells may play an important role in the induction of immunological tolerance. In their studies, tolerance induction was studied in two groups of mice; both groups were thymectomized, lethally irradiated and bone marrow grafted. One group was also given thymocytes along with the bone marrow. Both groups were then given a large number of sheep red blood

cells (SRBC) over a thirty day period. Following termination of this tolerance induction schedule, neither group of mice could respond to an immunizing dose of antigen. However, if normal thymocytes were inoculated at the time of immunization, the mice that had been pretreated in the absence of thymocytes could respond as well as non-pretreated controls. On the other hand, mice pretreated in the presence of thymocytes were totally unable to respond to an immunizing dose of antigen even after the addition of further thymocytes. Thus the presence of thymus derived lymphocytes during the course of tolerance induction had created a milieu where normal thymocytes could not cooperate with pretreated bone marrow cells.

In a subsequent study (Gershon and Kondo, 1971), the immunosuppressive effect of the presence of thymocytes during antigen pretreatment was studied by adoptively transferring the spleen cells of the antigen pretreated mice to thymus-deprived chimeras. These spleen cells not only did not cooperate with normal thymocytes in the secondary hosts but they also prevented the cooperation of normal thymocytes with normal bone marrow cells. The abrogation of the cooperation in the secondary host was specific in that the addition of spleen cells did not affect the anti-horse red blood cell response.

The first description of suppressor cells in neonatal tolerance to antigens of the major histocompatibility complex (MHC) was provided by Dorsch and Roser, 1974. In

their experimental system, adult rats were exposed to a sublethal dose of irradiation that reduced but did not eliminate reactivity to skin allografts. The inoculation of syngeneic lymphoid cells from normal rats restored normal reactivity, but the same number of cells from classically tolerant rats resulted in the essentially permanent acceptance of the test grafts in over 60% of the recipients. Third party allografts were rejected, indicating specificity of the suppression, and all of the suppressive activity of spleen and lymph node cell suspensions were found in primarily T cell suspensions obtained by thoracic duct cannulation (Dorsch and Roser, 1975).

Since these initial reports, there has been a flood of reports of suppressor cell involvement in most modalities of alloreactivity both in vitro and in vivo. Adoptive transfer experiments have now been repeated by many laboratories, sometimes with minor technical modifications, and have amply confirmed that suppressor cells are of major importance in the maintenance phase of transplantation tolerance (Roser et al., 1983).

In vitro generation of suppressor cells

Most experimental systems have utilized the mixed lymphocyte culture reaction (MLC) for the generation and detection of suppressor cells. Some of the methods for inducing and assaying in vitro generated suppressor cells are shown in Table I. The one way MLC reaction employed

TABLE I.

Induction and assay of in vitro generated suppressor cells.

Author	Induction in Primary Culture	Assay in secondary Culture
Shou - 1976	Mitogenic: ConA	MLC
Kurnick - 1976	Mitogenic: PHA	MLC
Hirschberg - 1977	a. Autologous MLC b. Allogeneic MLC	MLC MLC
Ferguson - 1977	a. Unstimulated culture b. Allogeneic MLC	MLC-CML MLC-CML
Smith - 1979	Autologous MLC	MLC, MLC-CML
Frey - 1980	Allogeneic MLC	MLC

for the generation of suppressor cells, usually referred to as primary MLC, consists of two allogeneic cell populations, responders and stimulators. The stimulator cells are inactivated by treatment with either mitomycin C or x-irradiation. At the end of a given period of incubation, the cells are harvested, washed, irradiated and added to a fresh one way MLC, which is referred to as the secondary MLC. The secondary culture, therefore consists of three cell populations, responder, stimulator and regulator (cells from the primary MLC). Usually, the regulatory cells are syngeneic to the responder cells and the stimulator cells are the same in both primary and secondary cultures. The inhibitory activity of the regulator or suppressor cells on proliferative or cytotoxic responses is assayed in the secondary MLC (Rich et al., 1975).

The criticism of this type of assay was that the suppressor cells from the primary MLC were cytotoxic cells and hence suppression of the generation of cytotoxic lymphocytes (CTL) was due to elimination of the stimulator cells. The experimental data, however, does not support this. Sinclair et al. (1976) has shown that preincubation of cells from primary MLC cultures with stimulator cells prior to the addition of responder cells gave less suppression of the generation of CTL in the secondary MLC, than the preincubation of primary MLC cells with responder cells. In addition, increasing the number of stimulator cells did not reduce suppressor cell activity, whereas altering the

concentration of responder cells had an effect, with stronger suppression seen with low numbers of responders (Triutt et al., 1977).

Though cytotoxicity does not explain the suppression seen, the exact mechanism of suppression in this system is still not clear. The suppressor cells may interact with responder, stimulator cells, or both. This interaction could be a direct cell to cell interaction or it could be mediated by a soluble factor (Rich et al., 1975). Evidence to support each of these mechanisms has been obtained by various groups, suggesting that more than one type of suppressor cell is operative in this system (Ferguson et al., 1977, Hanes et al., 1978).

The characteristics of the suppressor cells have varied with the method of induction. Those induced in autologous MLC are nonspecific and require a proliferative phase in secondary cultures, and they are sensitive to radiation and mitomycin C (Smith et al., 1979; Sakane et al., 1979). Suppressor cells induced by alloantigeneic stimulation are fairly well differentiated, have some specificity and are less sensitive to irradiation (Frey, 1980, Kovithavongs et al., 1982).

Suppressor cells in animal models of transplantation tolerance

The suspicion that transplantation tolerance was not due only to clonal deletion but involved positive

suppression of the rejection response came as the result of the experiments of Dorsch and Roser in 1974. Using an adoptive transfer model, they showed that inoculating cells from syngeneic tolerant animals delayed or prevented skin graft rejection in irradiated recipients. With a similar type of assay, six other laboratories have also demonstrated suppressor cells in classically tolerant mice and rats (Table II).

Further research has concentrated on the phenotype and functional attributes of the suppressor cells. Phenotype - Roser and Godden (1983) have shown that potent suppression is mediated by long lived, thymus derived radiosensitive cell populations, more than 99%. of which are T cells. One useful marker that differentiates suppressor cells from other peripheral T cells is their adherence to and recoverability from nylon wool (Holan et al., 1978). The monoclonal antibodies for helper and cytotoxic/suppressor subsets in the rat, however, were not found to be useful as markers in the suppressor cells of transplantation tolerance (TT). Evidence from more than one laboratory suggests that there may be several types of suppressor cells involved in TT. Gorczynski (1979) has presented evidence for a suppressor which prevents maturation of cytotoxic precursor cells (CTLP) to cytotoxic effector cells (CTL) and also for another suppressor that prevents the generation of CTL from bone marrow stem cells.

TABLE II.

Suppression in Animal Models of Transplantation Tolerance

Authors	Species	Recipient Treatment	Assay of Suppression
Dorsch (1975)	Rat	750 rads	S.G. survival
Holan (1977)	Rat	400 rads	S.G. survival, MLR
Gorczynski (1978)	Mouse	950 rads	CML
Smith (1980)	Rat	750 rads	S.G. survival
Vegh (1980)	Mouse	ALG	S.G. survival
Streilein (1981)	Mouse	250 rads	S.G. survival

S.G. - skin graft

Specificity

In general, experiments that have successfully demonstrated suppressor cells from transplant donors have found that the specificity of the suppressor cells is identical with the specificity of the toleragen (Smith et al., 1980), (Streilein et al., 1981). Exceptions to this, however, have been reported by Holan et al. (1981) who showed that B10.A(5R) mice tolerant to B10 showed cross reactive tolerance to B10.A(4R) skin grafts, and that the tolerant cells when cultured released a soluble factor that suppressed all MLR reactions tested, even where both responder and stimulator cells were fully allogeneic with B10.A(5R).

Antigen dependence

Since the initial experiments of Lubaroff et al. (1970), it appears convincing that transplant tolerance persists only as long as the antigen persists. Roser et al. (1980) showed that elimination of chimeric cells bearing antigen from adoptively transferred inocula of tolerant cells eliminates suppression, and conversely the immunization of tolerant radiation chimera donors with antigen greatly enhances suppression.

Variability

Transplantation tolerance demonstrated by in vitro and in vivo assays appears to be a highly variable state and

this probably is partly the reason for disparate results obtained by different workers. In certain strain combinations, attempts at induction of tolerance results in a minority of injected animals with complete tolerance, while the majority show failure of tolerance induction and even immunity (Streilein et al., 1979).

Analysis of cells of tolerant animals by adoptive transfer reveals considerable variation in suppressive potency from one donor to another and analysis in MLC and CML assays shows suppression in some hands (Hasek et al., 1979) but not in others (Gruchalla et al., 1982). Even where tolerant animals are screened for both failure to reject grafts and negative MLR or GVH responses, individual animals can be either highly suppressive or nearly negative in suppressor assays (Dorsch et al., 1982).

This characteristic of extreme variability may, of course, indicate that there is no single mechanism underlying transplantation tolerance, but that the tolerant state can result from a variety of mechanisms. Alternatively, it may indicate that the suppressor mechanism in transplant tolerance is a complex interacting network, certain components of which remain to be characterized.

Suppressor cells in clinical transplantation

The long term survival of allografted kidneys in patients receiving minimal doses of immunosuppressive drugs, was initially felt to be produced by enhancing antibodies.

Experimental data supporting this concept has been published by Hattler et al. (1972) and Bach et al. (1972). Since these initial reports, there have been quite a few papers published, using various methods of assay, attempting to delineate the mechanism involved in tolerance in clinical transplantation. See Table III.

Hattler et al. in 1972, using the MLC assay, found that the MLC responsiveness of the recipient to the donor in these patients was not depressed, however, it could be suppressed using the recipients' sera. They further identified the inhibiting factor as being present in the IgG fraction and it became progressively more evident after two months of uneventful post-transplant follow up (Hattler et al., 1973). No effect was seen with indifferent IgG or with IgG from other transplant patients. In addition, in patients whose sera demonstrated blocking activity, eluates from the kidney biopsy were also found to show blocking activity in the MLC assay.

Bach et al., in 1972, also found some evidence for blocking in the recipients' sera. However, they found that the recipient-donor MLC following Tx was depressed even after extensive washings to remove any blocking antibody. Hence, they concluded that there existed a central tolerance of the host with depletion of specifically reactive clones, and in addition, blocking antibodies facilitated the tolerant state.

TABLE III.

Suppressor Cells/Factors in Clinical Transplantation

Authors	Experimental Model	Results
Hattler (1972)	MLC responsiveness	Normal
	MLC suppression by serum	Positive
Bach (1972)	MLC responsiveness	Decreased
	MLC suppression by serum	Positive
Quadracci (1973)	Cytotoxic activity against donor fibroblasts	Decreased
	Suppression by serum	Positive
Thomas (1977)	MLC responsiveness	Normal
	MLC suppression by serum	Positive
	CML responsiveness	Decreased
	CML suppression by cells	Positive (Nonspecific)
Liburd (1978)	CML responsiveness	Decreased
	CML suppression by cells	Positive
Thomas (1979)	CML responsiveness by adherent cells	Positive
Goulmy (1981)	CML responsiveness	Decreased
	CML suppression by cells	Negative
Charpentier (1982)	MLC responsiveness	Decreased
	MLC suppression by cells	Positive
Pfeffer (1983)	CML responsiveness	Decreased
	CML suppression by cells	Negative

MLC and CML responsiveness refer to response of recipient against specific donor.

MLC and CML suppression refer to donor-specific tests suppressed by addition of recipients' serum or cells.

Quadracci et al. (1973) used a different system which measures the ability of specifically sensitized recipient lymphocytes to kill donor target cells, and the blocking of the cytotoxic effect by recipient serum. The target cells used were donor fibroblasts obtained from skin biopsies at the time of transplantation. Recipient lymphocytes and serum were tested at various times in the post-transplant course. They observed a steady decline in the number and frequency of reactive lymphocytes with a parallel increase in blocking activity, at various periods following transplantation. In the majority of the samples the loss of cellular immunity was preceded by the development of serum blocking factors.

Thomas and associates in 1977 investigated the lymphocyte responsiveness of long term renal allograft survivors in the MLC and the MLC-CML assays. Contrary to Bach's findings, they demonstrated that recipient cells were active in MLC against specific donors, indicating that donor-reactive clones were present. However, in the MLC-CML assay, the recipient cells were unable to generate donor specific cytotoxic cells, although they were capable of producing effector cells which could kill third party targets.

The majority of the recipients' sera demonstrated significant MLC blocking activity, even with serum dilutions of 1/5 to 1/500. In some preliminary experiments, they tested the effects of recipient lymphocytes on the induction phase of third party CML responses to the specific donor.

Their initial experiments showed a suppressive effect of CML non-responder lymphocytes, but in most cases they were not specific for donor cells, depressing also the CML response between control cells. In a subsequent study (Thomas et al., 1979), they demonstrated donor-specific suppressor cells, and that the suppressive activity could be abrogated by the removal of adherent cells, most of which were monocytes or macrophages.

Liburd et al., in 1978, were the initial workers to demonstrate a donor-specific suppression in the CML assay system. They compared the in-vitro CML induced in normal cells to mitomycin treated donor cells, with and without additional mitomycin treated recipient cells. They demonstrated donor-specific suppression in eight patients. Further studies suggested (Liburd et al., 1979) that the suppressor cells belonged to the T cell subset rather than the macrophages, as suggested by Thomas et al. Suppressor cell activity has also been demonstrated despite well documented acute rejection crises, raising the question of the role of suppressor cells in determining the clinical outcome of the transplant (Dossetor et al., 1981). The consistent finding of suppressor cells in HLA identical live donor transplants suggests that HLA sensitization is not required for suppressor cells to emerge following allografting, and it resembles the generation of suppressor cells in an autologous MLC reaction.

Goulmy et al, in 1981, reported on their studies on cytolytic potential (CML assay) and proliferative capacity in sixty-five donor-recipient combinations, twenty of them studied longitudinally with pre- and post-transplant samples. They found that patients with functioning grafts and with adequate follow up, over two-thirds became negative (<10% lysis) in the CML assay against the splenocytes of the kidney donor, although they were positive in this test with the lymphocytes from random donors. In half the patients with a negative CML test against specific donor splenocytes, 60 days or more post-transplantation, earlier samples were positive suggesting induction of donor-specific CML non-reactivity. Results of the MLC assay showed that if after transplantation antidonor MLC reactivity diminished, it diminished also against random donors. This is in contrast to the antidonor CML reactivity after transplantation, which seemed to be donor-specific. In five patients, a high donor-specific CML reactivity coincided with a reversible rejection crisis. They looked for suppressor cells in seven patients, using a coculture of pre- and post-Tx recipient cells with irradiated donor cells, but were able to demonstrate donor-specific suppression in only one out of the seven studied.

Agostino et al. (1982) reported on the suppression of third party MLC assays using lymphocytes from normal individuals, uremic patients and allograft recipients as putative suppressors. They found that lymphocytes from renal

allograft recipients displayed the greatest suppressive activity, with the frequency of tests displaying positive MLC suppression using $1x10^5$ lymphocytes as putative suppressors, was 90% for allograft recipients, 60% for renal failure patients and 28% for normal individuals. They also found a higher frequency of positive MLC suppression in patients who were given more than five blood transfusions as opposed to those who received less. Their fractionation experiments indicated that suppression of the MLC appeared to be mainly dependent on an esterase (+) adherent cell.

Charpentier et al. (1982), using the MLC assay between the recipient and donor, demonstrated a low responsiveness against the specific donor in cases of good graft tolerance, but this was absent during rejection and in control samples. For demonstration of suppressor cells, they used post-transplant recipient lymphocytes and added it to the same person's pre-transplant cells stimulated by the specific donor cells. They observed a donor-specific suppression of the proliferative capacity of pre-transplant recipient cells against the donor. Apparently, this was the initial study which demonstrated in a large group of patients, that donor-specific suppressor cells could also act at the proliferative cell level. They also demonstrated that there was no evidence of accelerated kinetics in the MLC assay to explain the low reponse and that the putative suppressor cells had to be added within the first 2 days of culture for the suppression to be observed.

In a subsequent study (Charpentier et al., 1983), they were able to isolate and characterize suppressor cells using monoclonal antibodies and cytofluorometric separation. The primary separation was into OKT8 reactive and non-reactive populations using the murine monoclonal antibody antiOKT8 (Reinherz, 1980). In an MLC assay using specific donor cells as stimulators, the proliferation of OKT8+ cells was depressed, whereas OKT8- cells showed a normal response. With mixing experiments, they showed OKT8+ cells exerted a suppressive effect specific to the donor-recipient pair on the proliferative response of the pre-transplant lymphocytes; conversely, the OKT8- cells exhibited an enhancing effect in a similar setup.

Pfeffer et al (1983) carried out a series of experiments in patients with well functioning kidney grafts. They also demonstrated a strongly reduced donor-specific cell mediated cytotoxicity in patients with normally functioning allografts from HLA-haplotype mismatched related donors. To explain the possible mechanisms involved in the specifically reduced cytotoxic response, they assessed, in vivo and in vitro suppressor cells, kinetics and varying effector:target ratios in the CML assay, the production of lymphokines and the effect of exogenous interleukin-2 (IL-2) on the CML assay.

In vivo formed suppressor cells were assayed in a system using the HLA identical sibling as responder, the specific donor as the stimulator and fresh mononuclear cells

from the recipient as the putative suppressor. Pooling the results for six patients tested, they observed a mean suppression of 20% in the proliferative assay (MLC system) and 4% in the cytolytic assay (CML system) (Pfeffer et al., 1983b).

In vitro suppressor cells were induced by culturing responder and irradiated stimulator cells for six days, and tested for suppressor activity by adding these cells to a fresh MLC and CML induction cultures from the same cell donors. After 6 days of culturing the MLC and CML activity were tested and compared with those of control cultures without suppressor cells. The results showed that all the recipients were able to form moderate to strong suppressor cells in vitro to the same extent as controls when tested in both proliferative and cytotoxic assays.

Kinetic studies of the CML assay between the recipient and the donor showed no evidence of accelerated kinetics, and varying the effector:target ratio showed that the reduced cytotoxicity persisted at varying ratios as compared to the control. Soluble growth factors were assayed in the supernatant of the recipient and donor cultures, by addition to a third party autologous MLC or IL-2 dependent cell lines. No correlation was found between the strongly reduced CML and production of growth factors. Exogenous IL-2 was added to see if higher concentrations would increase the cytotoxicity of the recipient toward the donor. No clear increase of the recipient toward donor cytotoxic response

could be found while the cytotoxic response of the sibling toward the donor increased 1.5-2 times. With these results, the authors conclude that the weak donor-specific cytotoxic response is caused by an in vivo depletion of cytotoxic cells.

Hence, the variability of results obtained in assaying for suppressor cells in clinical transplantation is similar to the results in the animal experiments, and is difficult to explain other than by saying multiple mechanisms of varying degree are operative in the production of transplantation tolerance.

II. MATERIALS AND METHODS

A. Preparation of Peripheral Blood Lymphocytes (PBL)

The method of Boyum (1968) with modifications, was used to prepare mononuclear cells from peripheral blood. Seven ml tubes of heparinized venous blood were collected and centrifuged at 1500 rpm (500xg) for 7 minutes. The plasma was removed and 1-2 ml of buffy coat was collected, pooled and diluted with approximately equal volumes of RPMI 1640. Five ml of this buffy coat suspension in RPMI was layered over 5 ml of Ficoll-Hypaque (F-H) (S.G. 1077) in 17 x 100 mm clear Falcon plastic tubes and centrifuged at 2000 rpm (800xg) for 20 minutes at 18-20 °C. The layer of cells at the interphase was collected with a pasteur pipette and transferred into another plastic tube, washed 3 times and resuspended in RPMI. Viability was estimated by dye exclusion with trypan blue (0.01% mixed in 1:1 ratio with a cell suspension) and counting in a hemocytometer. The cells were resuspended in RPMI with 10% pooled human serum (PHS) at the required cell concentration, and kept at 4 °C until use.

B. Separation of Different Subpopulations of PBL

Preparation of T Lymphocytes by Sheep Erythrocyte(SRBC) Rosetting

The method of Pellegrino et al (1975) was used with modifications. This technique requires the preparation

of amino ethyl isothiouronium hydrobromide (AET) treated SRBC and SRBC absorbed fetal calf serum (FCS).

1. Preparation of AET Treated SRBC

SRBC collected in Alsever's solution and stored less than 2 weeks in the cold were used. 2-5 ml of SRBC in Alsever's solution is washed twice with normal saline, removing the supernatant fluid and the top layer of cells with each wash till 0.2 ml of packed SRBC is left at the end of the washes. To this was added 8% AET (Sigma Chem). This mixture was incubated at 37 °C for 20 minutes in a shaking water bath. These cells were then washed 3 times with RPMI 1640. 0.2 ml of the SRBC were resuspended in 8.0 ml of RPMI-1640 and 2 ml of FCS which had been absorbed with SRBC.

2. Preparation of SRBC absorbed FCS

FCS was absorbed with SRBC for use in rosetting to remove naturally occurring anti-SRBC antibodies (Fauci and Pratt 1976). FCS was first decomplemented at 56 °C for 30 minutes then mixed with SRBC at a ratio of 2:1. The mixture was incubated at room temperature overnight. The tubes were spun at 1500 rpm (500xg) for 10 minutes, the serum separated and stored at -70 °C for future use.

3. Rosetting technique and separation of rosetted cells

PBL were prepared as described above and adjusted to a concentration of 5.0 x 10^{4}/ml. Equal

volumes of these PBL and AET treated SRBC were mixed. The suspension was spun at 1000 rpm for 5 minutes and then stored at 4 °C for 30 minutes. The cells were resuspended gently and then layered on Ficoll. The tubes were spun for 20 minutes at 2000 rpm at 4 °C. Rosetted T cells and free SRBC settled to the bottom of the tube while B cells and monocytes remained at the interphase. The cells at the interphase were aspirated into a separate tube and the T cells in the pellet were recovered by hypotonic lysis. 9.0 ml of 0.84% NH_4Cl was added to the pellet of rosetted T cells mixed and the tubes then spun at 1500 rpm for 7 minutes. The procedure is repeated till all the SRBC undergo lysis. The T cells are washed twice with RPMI-1640 and suspended in 10% PHS.

Preparation of T Cell Subsets

Monoclonal Antibodies (MAb) which mediate complement dependent cell lysis were used to prepare T cell subsets (Kovithavongs et al., 1984). These monoclonal antibodies were produced in Dr. John Hansen's Laboratory at the Fred Hutchinson Cancer Research Center in Seattle. The specific M Abs used and their reactivity are

1. MAb 35.1 - pan T
2. 66.1 - T helper
3. 51.1 - T suppressor/cytotoxic

(Hansen JA, 1982).

Preparation of T helper and suppressor/cytotoxic subsets

T cells are prepared by SRBC rosetting, to an aliquot of 5 x 10^4 T cells are added 0.1 ml of M.Ab. 51.1 (1:100 diln) and 0.1 ml of 50% Low-Tox-H rabbit complement (Cedarlane, Hornby, Ontario). The mixture was incubated for 2 hours at 37 °C in 5% CO_2 after which the cells were washed twice with RPMI then counted with Trypan blue. The viable cells remaining are the T helper enriched population.

A similar procedure was carried out, substituting M.Ab. 66.1 for 51.1 to obtain a T suppressor-cytotoxic enriched population.

The purity of each subpopulation was assessed using complement dependent cytoxicity in a ^{51}Chromium release assay. The percent killing obtained demonstrated a 90% pure population of T helper and T suppressor/cytotoxic cells.

C. Culture Medium

Medium RPMI 1640 (Gibco) supplemented with 100 u of penicillin per ml, 100 µg of streptomyin per ml, 0.25 µg Amphotericin per ml, and 10% pooled human serum (PHS) was used routinely for culturing.

The pooled human serum was obtained from male nontransfused donors selected for nonreactivity for HbsAg, absence of atypical Red Blood Cell antibodies and absence of cytotoxic HLA-ABC locus antibody. (Medical specialities

Laboratory Inc. Boston). The PHS was decomplemented in a 56° water bath for 30 minutes before use. Washing procedures were performed with RPMI 1640 without the addition of serum.

D. Freezing and Reclaiming of Lymphocytes

PBL was first prepared as described, and suspended at a concentration of 20 x 10^6 per ml. The cell suspension is placed in the freezer for at least 15 minutes to cool it down to 4 °C. A 20% solution of dimethylsulfoxide (DMSO) (BDH Chemicals) in 15% PHS in RPMI media is prepared. The DMSO is added to the media and not the other way around, and the final solution is cooled in the freezer for 15 minutes at 4 °C prior to use.

The 20% DMSO solution is added drop by drop to an equivalent volume of the cell suspension over a period of 7-10 minutes. After gentle resuspension 1 ml aliquots are placed in Nunc freezing vials. (InterMed, Roskilde Denmark). These vials are immediately placed in an uncovered rack in a -80 °C deep freezer. After one hour or for convenience the next day, the tubes are transferred for storage in the vapor phase of a nitrogen freezer (-190 °C).

For reclaiming of frozen lymphocytes, the vials are placed in warm water and the cells thawed. The cell suspension is transferred to plastic tubes and an equal volume of RPMI added. More RPMI is added with mixing over a period of 7 minutes to reach a final volume of 12 ml. The cells are then washed twice and then resuspended in 10% PHS

in RPMI at the desired concentration, prior to use in both the assays described below (Figure 1).

E. Cell Mediated lympholysis inducibility (CML) assay in Microtitre Plates

The technique of Hirschberg, Skare and Thorsby (1977) was used with minor modifications.

Culture Protocol

Lymphocytes from responder and stimulator populations were prepared as described above. After washing, they were cultured in culture medium at 37 °C in 5% CO_2.

The effects of culture period and numbers of lymphocytes were investigated to establish optimal conditions.

The sensitizing mixed lymphocyte cultures were established in the wells of round bottomed micro culture plates (Linbro IS MRC 96, New Haven, Conn.) employing 1 x 10^5 responding lymphocytes in combination with an equal number of irradiated (3000 rad) autologous or allogeneic stimulating cells in a total volume of 0.2 ml. Triplicate cultures were set up for each combination. These cultures were incubated for 6 days.

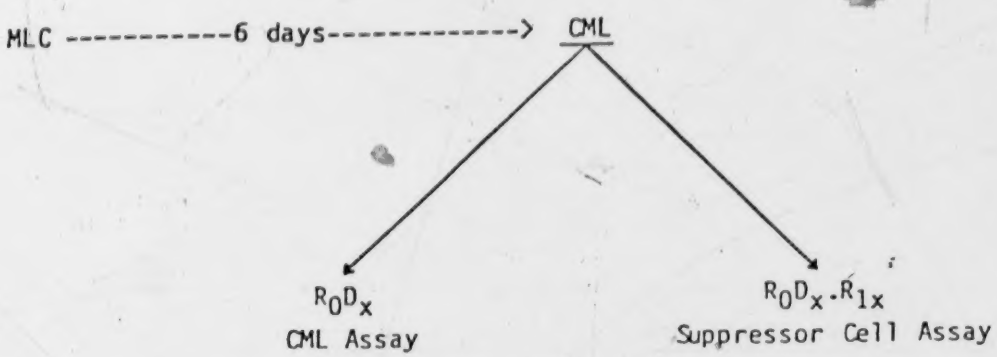
Target cells

5-7 x 10^6 lymphocytes were incubated in tissue culture tubes with 9 ml of 10% PHS in RPMI and 1 ml of 10% PHA.

FIGURE -1

Principle of CML and Suppressor cell assay

1. CML responsiveness of R_0, R_1, R_2, R_3 to stimulation by D or Y "CML assay"
2. CML inhibition by post transplant cells, i.e. inhibition of R_0D_x by R_{1x}, R_{2x}, R_{3x}: "Suppressor cell assay"



The PHA-treated target cells were incubated for 72 hours in 5% CO_2, 90% humidity at 37 °C. Eighteen hours before the target lymphocytes were harvested, 200 microcuries ^{51}Cr Sodium Chromate ($Na_2\ ^{51}CrO_4$, Amersham Lab) was added to each culture in the original volume and the cell pellet resuspended. After the 18 hour pulse, the cells were washed 3 times in RPMI 1640 and resuspended at a concentration of 8 x 10^4 lymphoblasts/ml.

Effector-target incubation

After 6 days of incubation of the sensitizing culture, the supernatant in each well of the microplate is aspirated with a multiple aspiration unit (Hamilton microsyringe Co., Cal. U.S.A.) so that approximately 0.15 ml remains in each well. Twenty-five lambda of the target cell suspension (8 x 10^4 lymphoblasts/ml) were injected using a 1 ml syringe and a Hamilton dispenser. The targets were injected directly into each culture, the force of the jet resuspending the cell pellet at the bottom of the well. After all the targets were injected, the microplate was lightly spun at 600 rpm for 1 minute and then incubated for an additional 6 hours.

Supernatant collection

The supernatant collecting system (Skatron A/S, Lierbyen, Norway) consists of a plastic holder containing 48 cellulose acetate absorption cartridges and 48 glass fiber filter discs. The pattern of the cartridges and filter discs

in the frame corresponds exactly to the pattern of the wells in the microplates. Using a simple press, the cartridges are aligned and pressed into the wells, the supernatant is absorbed into the cartridge and the cellular elements in the bottom of the well are trapped under the filter. The cartridges now containing most of the supernatant from the wells are transferred to special counting vials and counted in a Gamma scintillation counter.

Controls

In every experiment negative control wells with media alone or autologous cultures were set up for each target used. Positive controls were obtained by using 1% Hemolyte in the wells for each target.

Calculation of results

The spontaneous release was determined by adding targets to the media alone or the autologous culture wells and the maximum release from the hemolyte wells.

The percent specific ^{51}Cr release was calculated from the following formula:

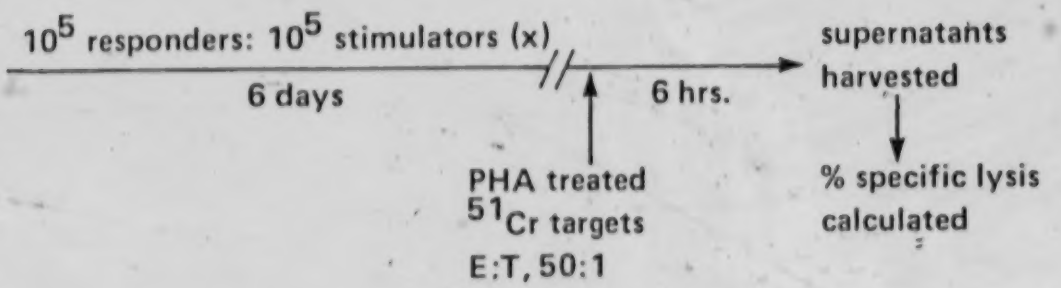
$$\% \text{ Release} = \frac{\text{cpm Exp.} - \text{cpm Control}}{\text{cpm Maximum} - \text{cpm Control}} \times 100 \quad \text{(Table IV)}$$

TABLE IV

CML and Suppressor Cell Assay - Technical Aspects

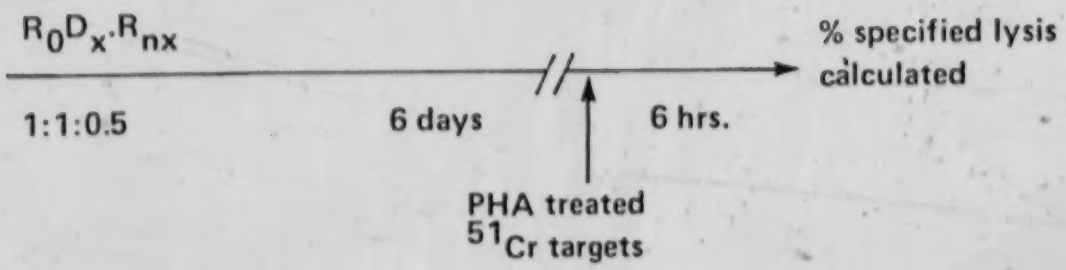
CML ASSAY

Microtitre plates, triplicate cultures set up



(x) – 3000r

SUPPRESSOR CELL ASSAY



D_x – 3000r
R_{nx} – 1000r

F. Suppression of CML Assay (Suppressor Cell Assay) in Microtitre Plates

The technique of Dossetor et al. (1981) with minor modifications was used in this project.

The effects of culture period, numbers of lymphocytes, radiosensitivity of putative suppressor cells were investigated to establish optimal conditions.

Culture protocol

The principle of this assay is to determine the amount of suppression obtained, by adding irradiated post transplant recipient cells, to a CML set up between the stimulator donor cells and responder pretransplant recipient cells. The suppressive activity of the post transplant recipient cells was tested primarily using unseparated PBL suspensions, though in limited experiments, T cell subpopulations separated as described earlier were used.

The sensitizing culture was set up with responder, stimulator and putative suppressor cells in a ratio of 1:1:0.5. 10^5 responders and 10^5 irradiated stimulators (3000 rads) with 5×10^4 irradiated suppressors (1000 rad) were incubated in a microculture plate for 6 days. Triplicate cultures were set up for each combination. The various cell combinations used in both the CML assay and the suppressor cell assay are shown in Table V.

The method of preparation of targets, effector-target incubation and collection of supernatant was identical to

that described under CML assay methodology.

Calculation of results

The percent specific chromium release was calculated for each cell combination as described earlier. Percentages of suppression were calculated with the formula.

$$\text{percentage suppression} = 1 - \frac{RoDx.Rnx}{RoDx.Rox} \times 100$$

(for explanation of letters, see Table V)

G. Patient Population

A total of 30 recipient-donor combinations were studied. Fifteen of these had normal renal functions at one year and they comprised Group I. The remaining 15, Group II, had severe rejection during the first year post Tx, requiring graft nephrectomy and/or maintenance hemodialysis within the one year period.

Lymphocyte samples

Serial samples were obtained from all the recipients, both pre- and post-transplant (Tx) and were designated accordingly; pre Tx (Ro), post Tx 10 to 30 days (R1), 31 to 90 days (R2), and 91 to 365 days (R3). Samples were also obtained from the specific donor (D) and a healthy control (Y).

TABLE V

Experiments and Controls Set Up For Each Recipient-Donor Combination

CML Assay		Suppressor Cell Assay	
Exp's	Controls	Exp's	Controls
R_0Dx	R_0Yx	$R_0Dx.R_0x$	$R_0Yx.R_0x$
R_1Dx	R_1Yx	$R_0Dx.R_1x$	$R_0Yx.R_1x$
R_2Dx	R_2Yx	$R_0Dx.R_2x$	$R_0Yx.R_2x$
R_3Dx	R_3Yx	$R_0Dx.R_3x$	$R_0Yx.R_3x$
			$R_0Dx.Yx.$

R_0 - pre-transplant recipient cells

R_{1-3} - post-transplant recipient cells

D - Donor

Y - unrelated normal

In a few experiments where pretransplant cells were inadequate, a normal control (A) was used. All the samples were cryopreserved in liquid nitrogen and reclaimed at the time of the study. The recipient samples tested were all from patients with the graft in situ, and for an individual patient all the samples in both the assays were run simultaneously. The numbers of samples tested at the various time intervals, in both the groups are shown in Table VI.

Patients with normal renal function at one year-Group I

Fifteen donor-recipient pairs were investigated, with at least 3 post-transplant samples being studied per patient. Five patients received renal allografts from HLA haploidentical living related donors and the other 10 from cadaver donors. All the transplants were functioning at the time of the study and the renal function, as assessed by the serum creatinine, was normal at the end of the 1st post transplant year. Eleven patients were treated with cyclosporine and the other 4 with azathioprine. The patient group characteristics are shown in Tables VII a and b.

TABLE VI

Patient Population and Samples

Patients Total of 30 recipient-donor combinations:

Group I 15 – normal renal function at one per year

Group II 15 – severe rejection, requiring nephrectomy/HD within one year

Patient Samples Tested

	Pretransplant (Ro)	Post Transplant (days)		
		10-30 (R_1)	31-90 (R_2)	91-365 (R_3)
Group I	15	15	20	20
Group II	15	15	15	6

TABLE VII

a) Patient Group Characteristics

	GROUP I	GROUP II
Donors	10 CD, 5 LRD	15 CD
Mismatched 1	4	7
DR Ag's 2	4	5
Immunosuppression		
- CyA	11	9
- Aza	4	6
2nd Grafts	--	6
Mean time to graft failure	--	4 months
Graft Nephrectomy	--	11

b) Patient Group Characteristics - Renal Function

	GROUP I		GROUP II	
	R_1	R_2	R_1	R_2
No. on Dialysis	0	0	5	8
Grafts in situ	15	15	15	15
Serum Creatinine (Patients not on dialysis)	N	N	354 (n=10)	436 (n=7)

Patients with poor renal function at one year - Group II

The 15 recipients in this group had all rejected their grafts within one year post-transplant. Two to 3 post-transplant samples were obtained from these patients, the third post-transplant sample was not tested in all the patients, since some of them would have had a graft nephrectomy prior to the sampling date. All 15 patients had received cadaver donor kidneys, 9 of them were receiving cyclosporine and the other 6 azathioprine.

Five of the patients were on intermittent or regular dialysis at the time of the R1 sample and 8 were on dialysis at the time of the R2 sample, however all the samples tested were with the graft in situ. The details of the patient characteristics in this group are shown in Tables VII a and b.

Statistical methods

Tests for significant differences (p values) were performed using the students t-test. Unless specified otherwise these tests were paired. In all cases all the raw data (each of the 3 triplicates) were used.

III. RESULTS

A. Cell Mediated Lympholyis Assay

Kinetics

The kinetics of the various cell combinations used in the CML assay were assessed by using responder cells from recipients with normally functioning grafts and stimulator cells from the donor or the control. The cytotoxic effector cells were tested against appropriate targets on Day 3 and Day 6 of the primary CML.

Six recipient samples, both pre and post transplant, were assayed against cells of the donor and the control, and the results are graphically represented in Figure 2.

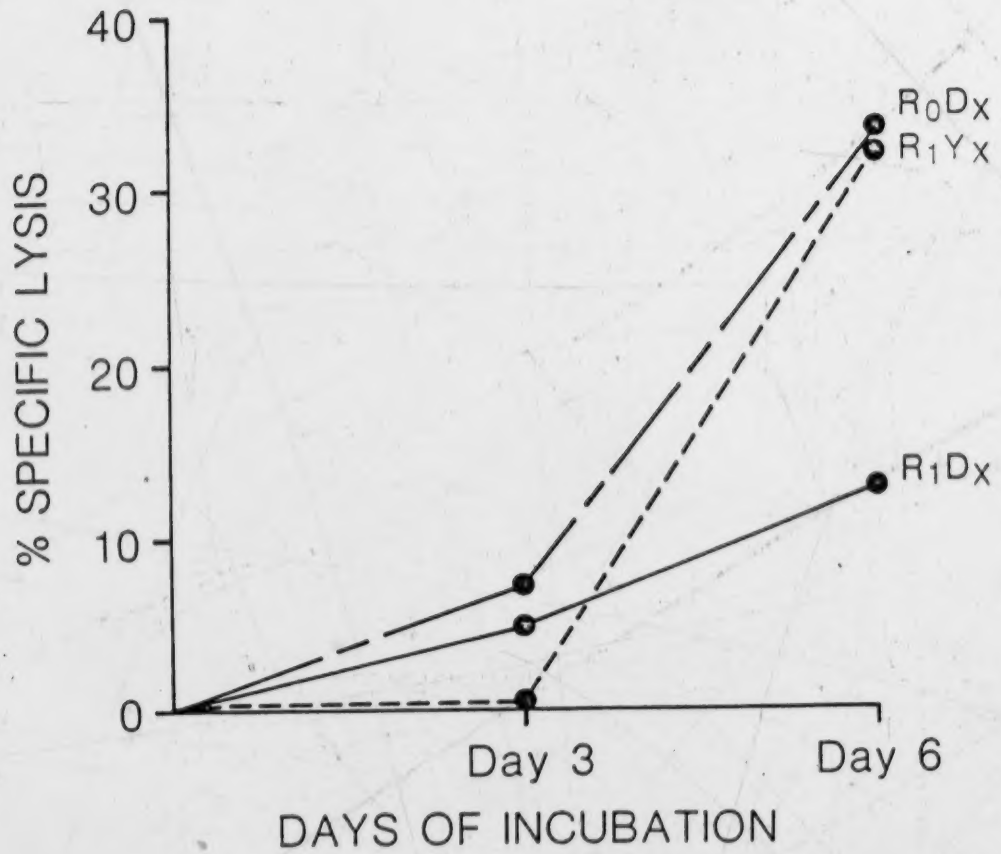
Pretransplant recipient cells when tested against cells of the specific donor or the control, showed a negative CML response at Day 3 and >30% specific lysis on Day 6.

Post transplant recipient cells when tested against cells of the specific donor showed a negative CML on Day 3 and only a 15% response on Day 6; however when tested against a normal control the Day 6 response was greater than 30% specific lysis.

These results suggested that there was no evidence for changes in the kinetics in either the pre or post transplant samples. The reduced cytotoxic response of the post transplant recipient cells against the specific donor,

FIGURE - 2

CML ASSAY - KINETIC STUDIES



could not be explained by accelerated kinetics, since the recipients' maximum cytotoxic response against both the donor and the control were observed on Day 6.

Varying effector:target ratio

The effect of varying the effector target ratio on the CML response was assessed using both pre and post Tx recipient cells. Responder cells from the recipient were titrated from 2 x 10^3 to 2.5 x 10^4 cells/well while the number of donor or control target lymphoblasts was kept constant at 2 x 10^3 cells/well. These combinations gave effector: target ratios of 100, 50, 25, and 12.5.

The results as shown in Table VIII, demonstrated that there was an increase in the percent specific lysis with increasing effector: target ratios for all the combinations tested. However, the post transplant recipient cells when tested against cells of the specific donor, showed a reduced cytotoxic response at all effector target ratios when compared to all the other combinations.

For all the experiments performed subsequently, the ratio used was 50:1, since this ratio gave adequate lysis and was convenient for use in respect to cell volume and numbers.

TABLE VIII

CML Assay - Varying Effector:Target Ratio

E:T ratio	% Specific Lysis			
	R_0Dx	R_1Dx	R_0Yx	R_1Yx
100	38	23	40	34
50	33	17	32	23
25	21	14	25	19
12.5	9	7	13	10

- Mean values from three separate experiments.

B. Suppressor Cell Assay

Radiosensitivity of suppressor cells

The optimum dose of radiation to be given to the putative suppressor cells was assessed in a series of experiments. In 5 patients, the post transplant recipient cells were given various doses of radiation, from no irradiation to 4000 rads, added to the primary CML culture and the percentage suppression calculated.

The individual results from the experiments are shown in Table IX. On pooling the results, the maximum suppression was seen with no radiation and progressively decreasing amounts of suppression are seen with higher doses of radiation, suggesting that these cells are radiosensitive. For all the subsequent experiments the putative suppressor cells were irradiated to 1000 rads prior to use, on the premise that this dose of radiation would prevent proliferation and possibly cytotoxicity against the donor cells (Pfeffer et al., 1983b).

Varying responder: suppressor ratio

The effect of varying the responder: suppressor ratio was assayed in 4 patients. Three responder: suppressor ratios were used, 1:0.25, 1:0.5 and 1:1. The number of stimulator cells was the same as the responder cells in all the experiments. The results of these experiments are shown in Table X. The 1:0.5 ratio of responder to suppressor

TABLE IX

Radiosensitivity of suppressor cells

Samples	No radiation	1000r	2000r	3000r	4000r
1	-6	-16	-8	ND*	-6
2	ND	-18	-9	+10	0
3	-19	-7	+4	+7	ND
4	-39	-31	-17	-20	ND
5	-21	+6	-8	0	-15
Mean suppression	-21	-13	-8	-1	-7
Control	-4	+2	-4	+7	+7

ND* - not done

- Negative values denote suppression, positive values, enhancement.

TABLE X

Suppressor Cell Assay - Varying Responder:Suppressor Ratio

Ratio Resp:Stim:Supp.	% Age Suppression FC	RG	RL	DS	MEAN'
1 : 1 : 0.25	-4	+3	-12	+2	-4
1 : 1 : 0.5	-12	-16	-16	-12	-14
1 : 1 : 1	-14	-12	-18	+1	-11

' - Best suppression seen with 1:1:0.5 ratio.

- Negative values denote suppression.

appeared to give the most consistent suppression, though the other 2 ratios also showed evidence of suppression. Subsequent suppressor cell assays were performed using a responder: suppressor ratio of 1:0.5.

Kinetics

Evidence for accelerated kinetics with the suppressor cell assay were looked for in a similar manner as for the CML assay. The results are shown in Table XI.

All combinations tested showed a negative CML on Day 3 and the maximum cytotoxic response was on Day 6. These results suggested that there was no evidence for accelerated kinetics in the suppressor cell assay.

The data shown in Tables IX, X and XI, for radiosensitivity, ratio of responder to suppressor, and kinetics are limited in numbers, primarily because of the difficulty in obtaining adequate samples of post-transplant lymphocytes. Hence, no statistical conclusions can be drawn from this data.

C. Results of CML Assay in Patients with Normal Renal Function - Group I

In the 15 patients with normal renal function at 1 year, CML assays were set up between the recipients pre and post Tx cells (R_1-R_3) and the specific donor (D) or the Control (Y). (Table V).

TABLE XI

Suppressor Cell Assay - Kinetics

Cell combination	% Specific Lysis			
	Pt. 1		Pt. 2	
	Day 3	Day 6	Day 3	Day 6
$R_eDx.R_ex$	6	27	7	38
$R_eDx.R_ix$	3	21	7	29
$R_eYx.R_ex$	0	52	5	44
$R_eYx.R_ix$	3.5	48	3	42

- No evidence of accelerated kinetics.

The pretransplant donor specific CML (R_0Dx) averaged 40% specific lysis, whereas the post transplant samples showed a reduced cytotoxic response with the mean specific lysis for R_1Dx being 20%, R_2Dx 18.5% and R_3Dx 17%. When tested against normal controls no significant difference in specific lysis, betwen pre and post transplant samples (R_0Yx vs. $R_{1-3}Yx$) could be demonstrated.

These results are shown in the form of a bar diagram in Figure 3, and demonstrates a donor-specific hyporesponsiveness in the post transplant samples.

Statistical analysis of the difference in responsiveness between the donor-specific pretransplant and the post transplant samples, by the students t test gave a t value of 5.81 and $p < 0.001$. Between the control pre and post transplant samples, no significant difference could be demonstrated.

D. Results of CML Assay in Patients with Poor Function - Group II

The CML assays between recipient, donor and control samples were set up as described earlier. In this group, the mean CML specific lysis of R_0Dx was 47%, post Tx R_1Dx remained elevated at 37, and R_2Dx at 41%. Control values, R_0Yx, R_1Yx and R_2Yx were 55%, 52% and 50% respectively. These results are depicted in a bar diagram in Figure 4.

Statistical analysis by the students t test, for the difference between the pre and post transplant samples, gave a p value of < 0.05 between R_0Dx and R_1Dx, whereas between R_0Dx and R_2Dx, as well as the control samples (R_0Yx vs. $R_{1,2}Yx$), there was no significant difference.

FIGURE -3

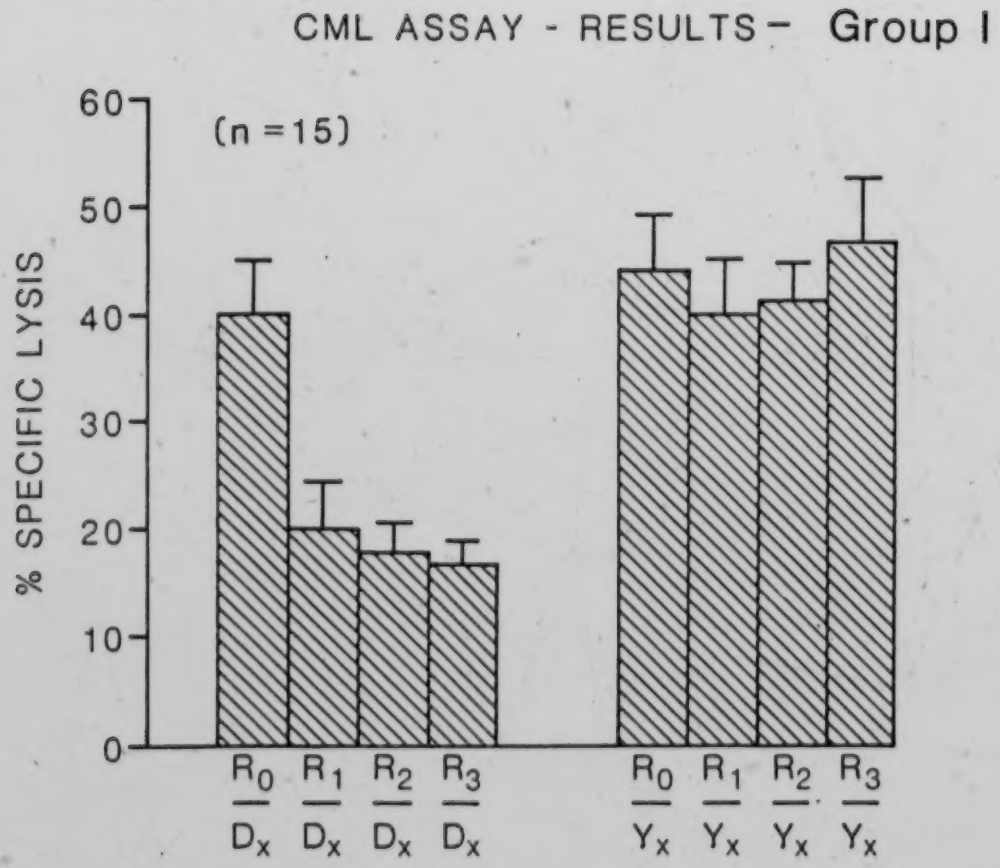
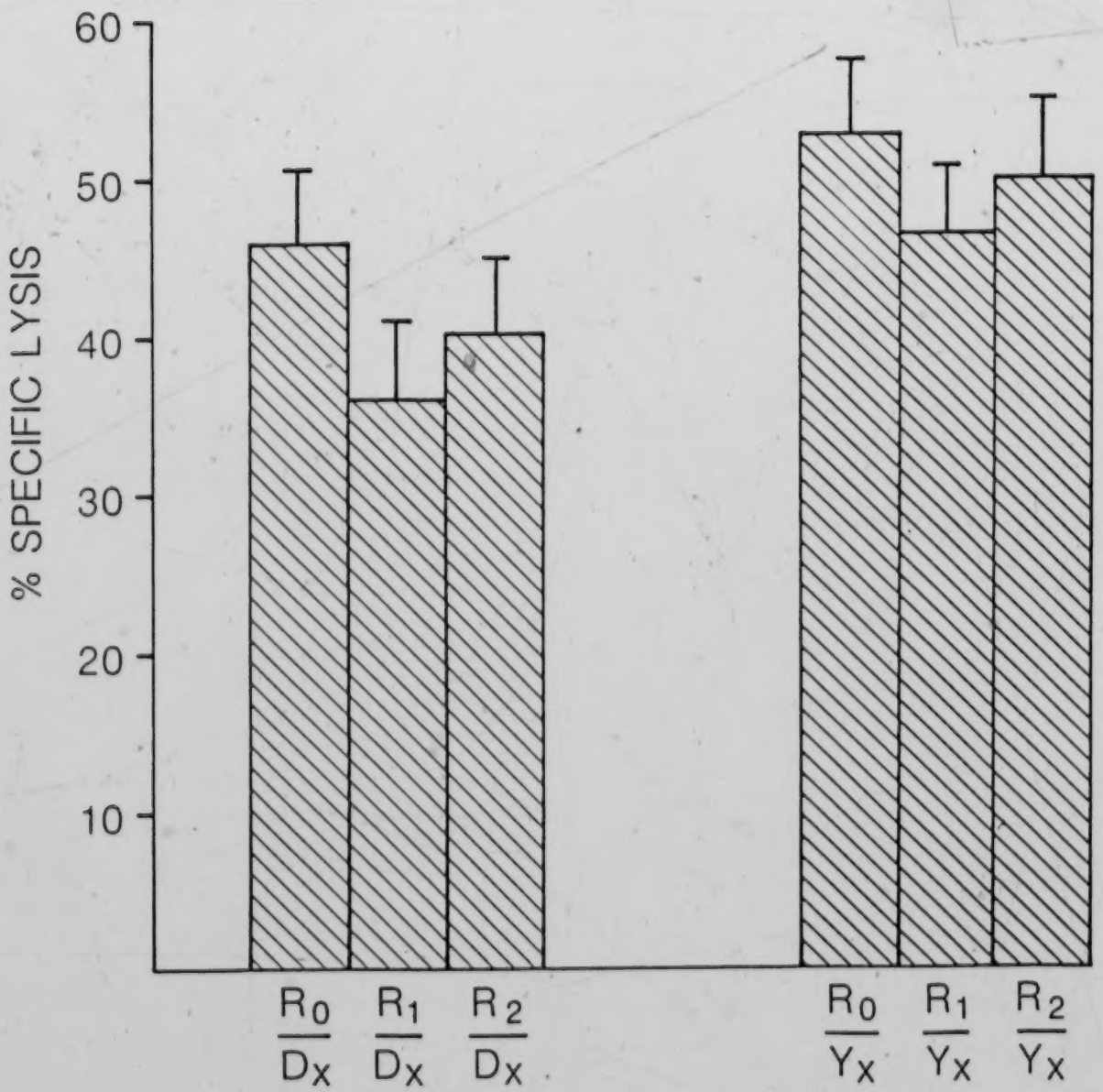

CML ASSAY - RESULTS - Group I
(n = 15)
% SPECIFIC LYSIS
60
50
40
30
20
10
0
R0/Dx
R1/Dx
R2/Dx
R3/Dx
R0/Yx
R1/Yx
R2/Yx
R3/Yx

FIGURE -4

CML ASSAY - RESULTS - Group II
(n=15)



E. Drop in CML Responsiveness Post Transplant - Comparison of Groups I and II

The percentage drop in CML responsiveness between the pre- and post-transplant donor-specific CML was compared in both the groups (Figure 5).

Group I patients showed a 50% or greater, drop in the mean CML responsiveness for all the post transplant samples at the 3 time periods. Group II patients on the other hand showed only a 20% drop at the time of the first post-transplant sample, and a 13% drop at the second post-transplant sample.

F. Results of Suppressor Cell Assay in Group I

The suppressor cell assay was set up using pre and post transplant recipient cells as the putative suppressors.

Statistical analysis was performed between the experiments and controls, using both the specific lysis values obtained as well as with the percentage suppression values calculated.

With the pretransplant recipient cells used as putative suppressors in the CML assay i.e. R_0Dx . R_0x, the percentage specific lysis was 58%, with post transplant cells as suppressors the specific lysis showed a drop, R_0Dx . R_1x 50% and R_0Dx . R_2x 49% (Figure 6). Test of difference (t) between the pre- and post-transplant samples gave p values of < 0.01.

FIGURE 5

% DROP IN CML RESPONSIVENESS POST-TRANSPLANT
COMPARISON OF GROUPS I & II

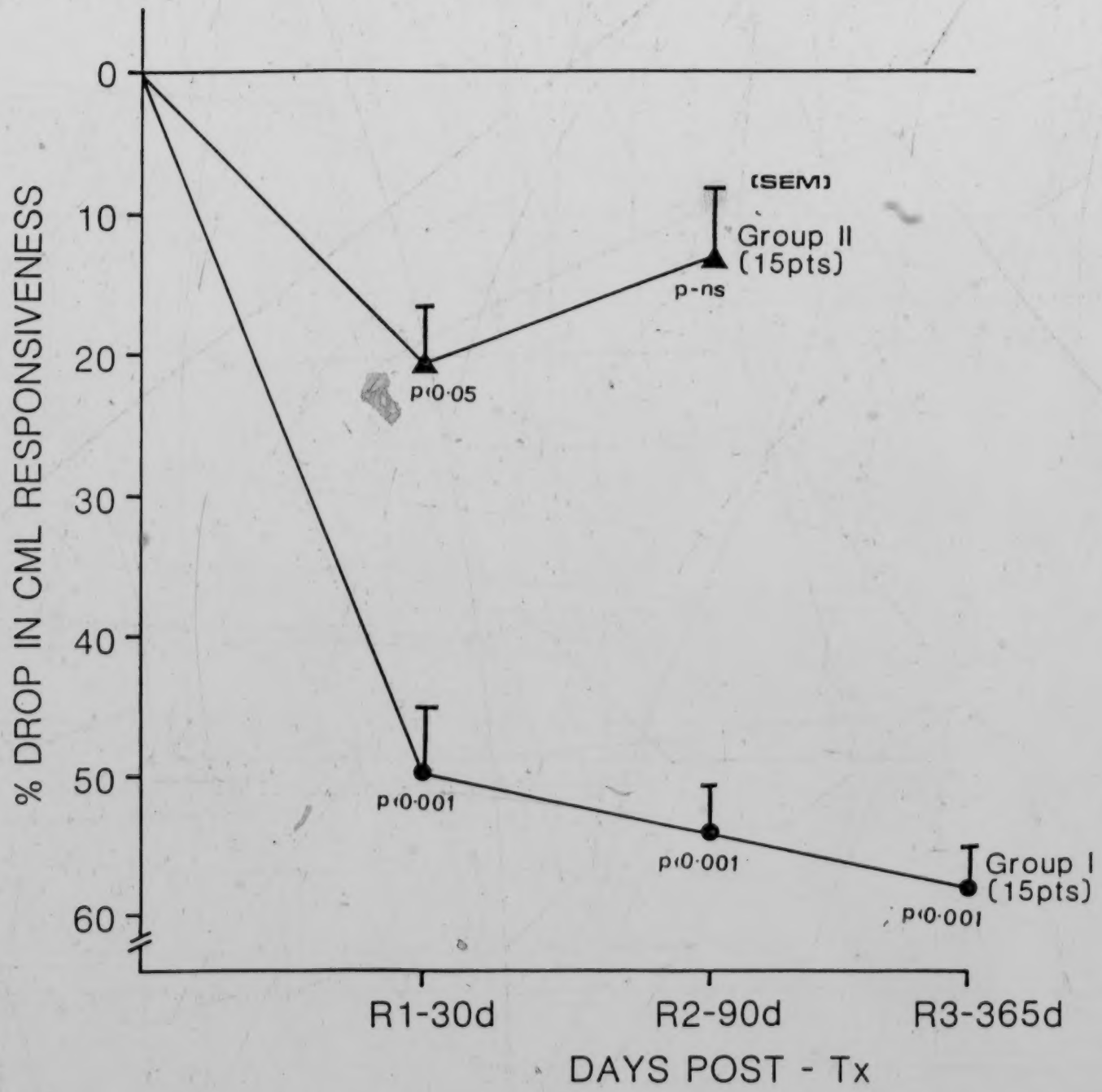
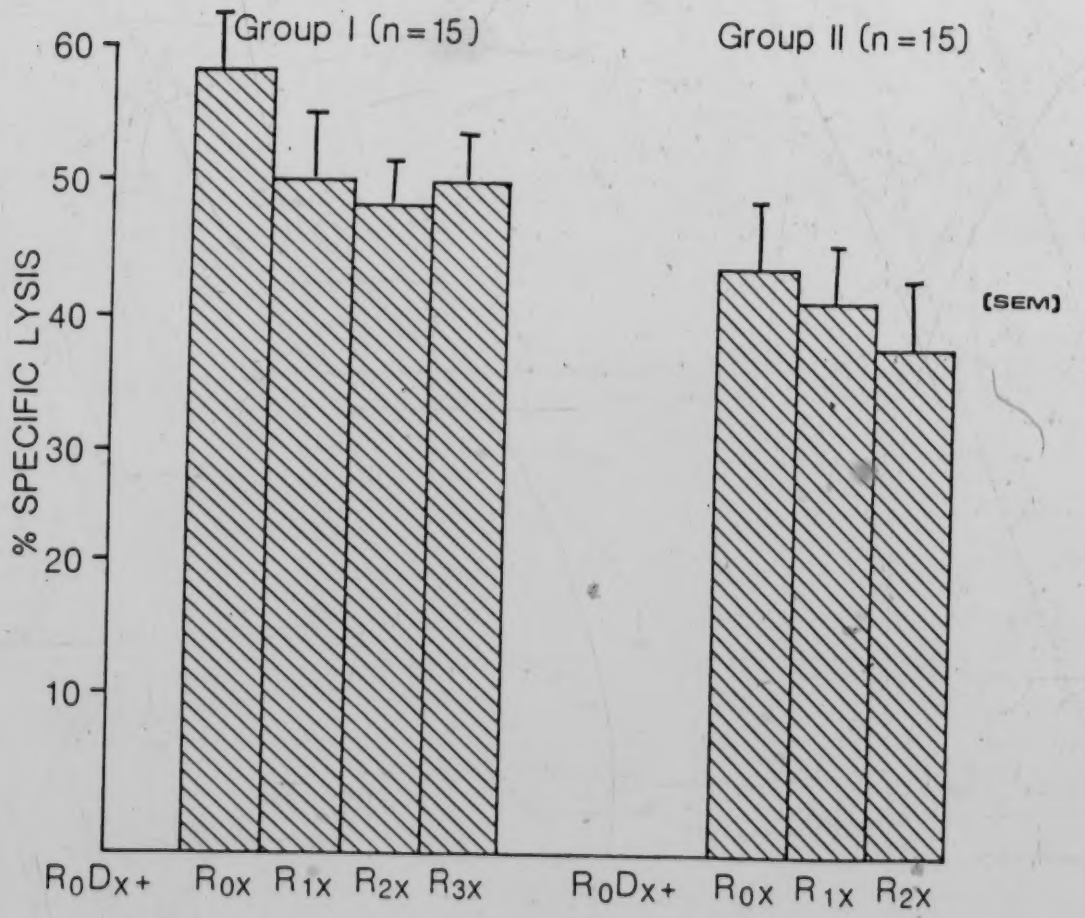


FIGURE 6

SUPPRESSOR CELL ASSAY - RESULTS



Percentage suppression calculated using the formula, [1 - $R_0Dx \cdot Rnx / R_0Dx \cdot R_0x$] x 100, gave a 12% suppression with the R_1 sample, the control showing 1% suppression ($R_1Yx \cdot R_1x$). R_2 showed a 15% suppression, with the control showing a 2% enhancement (Figure 7).

Hence, in Group I patients a statistically significant degree of suppression could be demonstrated.

G. Results of Suppressor Cell Assay in Group II

Figure 6 shows the effect of pre and post transplant recipient cells on the percent specific lysis of the CML set up as described earlier. The control pretransplant value $R_0Dx \cdot R_0x$ was 43.5%, compared with the post transplant values for $R_0Dx \cdot R_1x$, 41% and $R_0Dx \cdot R_2x$, 37.5% ($p < 0.025$).

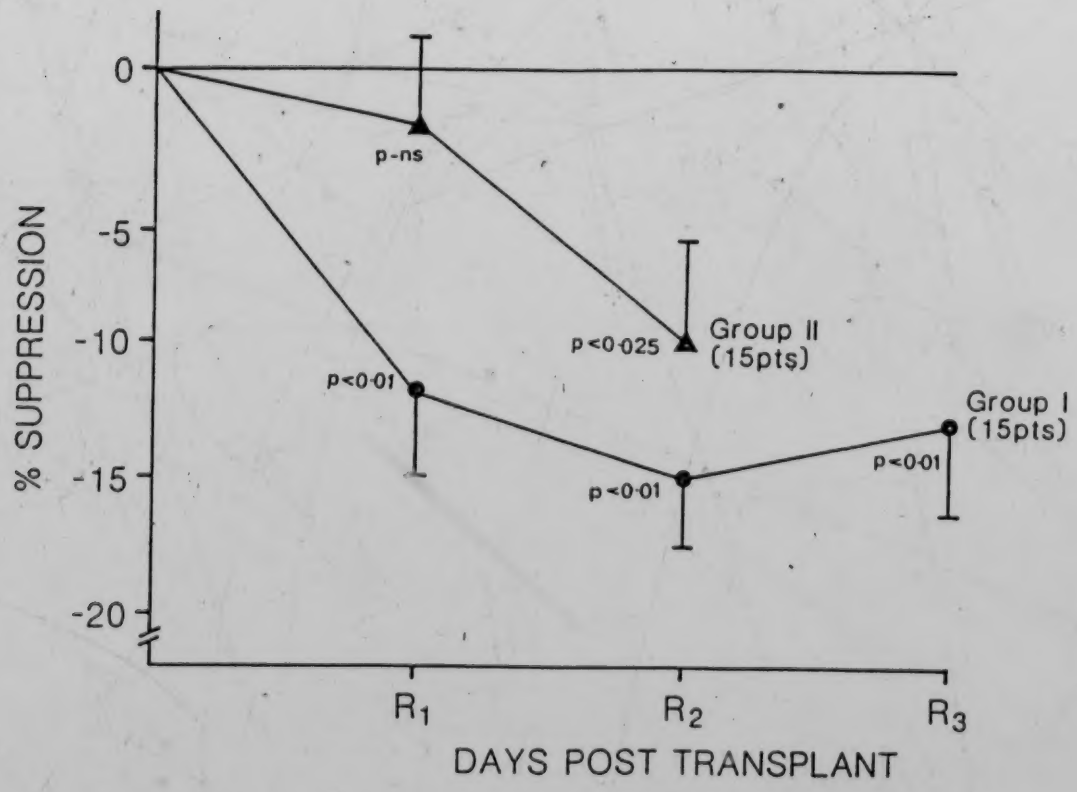
Percentage suppresson calculated using the formula showed a 2% and 10% suppression for $R_0Dx \cdot R_1x$ and $R_0Dx \cdot R_2x$, respectively. Control values $R_0Yx \cdot R_1x$ and $R_0Yx \cdot R_2x$ were 8% and 5% respectively. There was no statistically significant difference between the experimental and control values in this group.

H. Comparison of Suppressor Cell Assay in Groups I and II

Figure 7 shows a comparison of the percentage suppression in the 2 groups at the various time periods. Group I shows a significant suppression at all the time periods tested. In Group II, patients there was no

FIGURE -7

SUPPRESSOR CELL ASSAY - RESULTS



significant suppression at the R_1 sample, but the R_2 sample showed a significant suppression ($p < 0.025$) when compared with the pretransplant sample. However, on comparing the R_2 sample with another control ($R_0Dx \cdot R_2x$ vs. $R_0Yx \cdot R_2x$) the percentage suppression was not significant ($p < 0.4$).

I. Suppressor Cell Assay in HLA-Identical Renal Allografts

S.C. assay was carried out in 8 post transplant samples from 2 patients with normally functioning grafts. One of the patients range of suppression was from 3 to 26% and in the other from 4 to 11%. This was not significantly different from patients with non-identical grafts, suggesting that suppressor cells are not HLA restricted, and HLA sensitization is not required for suppressor cells to emerge following allografting.

J. Experiments with Lymphocyte Subsets

Lymphocyte subsets were prepared as described earlier and assessed for suppressive activity on the primary CML.

The results in 4 patients are shown in Table XII the mean suppressive capacity of PBL was 9%, which increased to 12% when T cells were used and 19% when T_4 cells were used. B cells and T_4 cells gave only 2 and 3% suppression respectively. These experiments though limited, suggest that the suppressive capacity lies in T cells and more specifically in the T_4 subset. However, the control experiments also showed 9% suppression when the T_4 subset was added, suggesting that these cells do have some non-specific suppressive activity.

Here too, the number of experiments are limited because of the difficulty in obtaining adequate numbers of post-transplant lymphocytes for these experiments. Hence, no statistical conclusions can be drawn from this data.

TABLE XII

Percentage suppression obtained using lymphocyte subsets in suppressor cell assay

	% Suppression / Subset Added						
Patient	PBL	T	B	T_4	T_8	T_{4+8}	Control*
DS	-11	-13	-5	-1	-22	-20	-13
LM	-18	-18	-13	-10	-23	--	-10
GP	-1	+14	+20	+10	-6	+8	-6
MD	-5	-30	-10	-11	-25	--	-8
MEAN	-9	-12	-2	-3	-19	-6	-9

* Control - R_0Yx + RT_0 cells.

- Negative values denote suppression

TABLE XIII

CML Assay - Statistics

Test of difference (t). cf to baseline

	RoDx	p
Group I	R,Dx	<0.001
	R,Dx	<0.001
Group II	R,Dx	<0.05
	R,Dx	N.S.

TABLE XIV

Suppressor Cell Assay - Statistics

Test of difference (t). cf to baseline

	RoDx.Rox	p.
Group I	RoDx.R_1x	<0.01
	RoDx.R_2x	<0.01
Group II	RoDx.R_1x	N.S.
	RoDx.R_2x	<0.025

TABLE XV

Clinical details of individual patients studied

Patients	Date of Tx	Donor	Matched DR Ag's	Graft No.	S. creatinine (mols/l) R,	R,	Pretransplant Blood Transfusions
Group I							
D.S.	1.12.82	LRD-Sibling	1	1	266	190	8
R.C.	2.1.83	Cadaver	0	1	137	198	7
M.D.	9.4.82	Cadaver	2	1	219	184	2
G.P.	13.4.83	LRD-Sibling	1	1	174	144	4
K.C.	17.11.82	LRD-Parent	0	1	86	83	12
W.B.	15.6.80	Cadaver	1	1	120	132	4
R.G.	17.2.83	Cadaver	1	1	159	209	5
M.G.	29.9.82	LRD-Sibling	1	1	159	157	2
B.K.	24.8.82	Cadaver	1	1	111	140	12
L.M.	24.8.82	Cadaver	1	1	201	115	2
F.P.	12.7.82	Cadaver	1	1	230	147	4
R.L.	4.6.81	Cadaver	1	1	161	174	2
D.S.	7.12.78	Cadaver	0	1	120	128	0
T.H.	27.4.83	LRD-Parent	1	1	170	108	13
A.W.	23.8.82	Cadaver	1	1	224	241	18
Group II							
J.B.	21.9.83	Cadaver	2	3	350	514	40
R.A.	9.9.83	Cadaver	0	2	512	n/a	5
E.G.	20.11.83	Cadaver	0	1	878	900	7
J.W.	20.11.83	Cadaver	0	1	244	297	8
A.S.	13.7.82	Cadaver	1	2	116	230	32
J.M.	3.10.83	Cadaver	1	1	1000	1200	0
P.E.	14.9.81	Cadaver	1	1	708	616	2
D.O.	17.2.83	Cadaver	1	2	700	634	10
S.K.	1.6.82	Cadaver	0	1	383	560	0
L.C.	4.1.82	Cadaver	1	1	600	678	2
W.B.	12.7.81	Cadaver	1	1	204	536	2
D.O.	25.10.82	Cadaver	0	1	236	400	2
L.B.	14.9.81	Cadaver	1	2	840	620	17
G.S.	17.11.82	Cadaver	0	2	800	894	11
M.S.	3.4.82	Cadaver	1	1	544	719	1

IV. DISCUSSION

The concept of immunological monitoring evolved shortly after clinical transplantation became accepted as a mode of therapy, with the recognition that graft rejection was due to an immune response and that agents responsible for the antidonor immune response might be detectable in vitro.

Over the last twenty years, four types of immunologic monitoring have developed:

1. assessment of the general level of host immunocompetence;
2. evaluation of the adequacy of immunosuppressive therapy;
3. search for secondary non-specific mediators of the immune response, e.g., fibrin degradation products;
4. detection of specific immunoreactivity toward donor cells, including the discrimination of donor-specific unresponsiveness.

Assessment of the general level of host immunocompetence can be done both pre-transplant, primarily as a predictor of graft function, and post-transplant as an indicator of rejection. Rolley et al (1978) noted that patients who were anergic to sensitization with dinitrochlorobenzene (DNCB) displayed better allograft survival than immunoreactive individuals. In addition, chronic pre-transplant Hepatitis B surface antigenemia appeared to improve one year renal graft survival. Kerman et al. (1980), using a battery of cell

mediated immune indices including the percentage of circulating active T cells, spontaneous blastogenesis and proliferative response to a panel of donors in one way MLC, found that strong responders displayed only a 39% one year cadaveric graft survival, compared with 72% in weak responders. Both groups were treated with azathioprine. However, the impact of pre-transplant immune status was mitigated by cyclosporine (Kahan, 1985).

Post-transplant, serial evaluation of general host immunocompetence has been used in an attempt to predict immune activation and rejection. Initial studies enumerated total T cells using the SRBC rosetting technique; with the advent of monoclonal antibodies to quantitate lymphocyte types, numerous studies have been published regarding their utility and limitations. Increased ratios of T_4 (helper-inducer T cells) to T_8 (suppressor-cytotoxic) subpopulations were correlated with allograft rejection by some workers (Cosimi et al. 1981, Ellis et al. 1982), but not by others (Carter et al. 1983, Burton 1984).

Donor-specific assays have logically attracted the most attention in immunological monitoring. Various humoral and cell mediated assays have been and are being used. These include complement dependent cytotoxicity (CDC), antibody dependent cell mediated cytotoxicity (ADCC), LMC, CML and MLC. Each of these donor-specific assays have their proponents and detractors as regards their usefulness in diagnosing rejection. Cross matching techniques using dye injection, ^{51}Cr release, or

fluorescence activated cell sorting (FACS) to measure CDC have demonstrated a high incidence of false negative tests when used with serial post-transplant patient sera. However, some investigators have found the CDC assay helpful. A more sensitive measure, the ADCC, again has been found to be useful in some hands (Gailunas et al., 1978), but not in others (Kovithavongs et al., 1978). Detection of the effector destructive mechanism by in vitro lymphocyte mediated cytolysis (LMC) is subject to numerous variables: length of killing time, detection methods for target lysis, effector-target cell ratio, and type of target pre-treatment, e.g., with PHA. However, under specific conditions, LMC activity correlated with clinical rejection episodes and appeared to display fewer false positive assays (Stiller 1976 et al., Kovithavongs et al., 1978).

This study has focused on the last mentioned mode of monitoring, using cell mediated donor-specific immunoreactivity as the index. Two assays have been used as described earlier: the CML assay and the suppressor cell assay. Both assays involve peripheral blood lymphocytes, long term preservation of cells in liquid nitrogen and radioisotope counting techniques, but the latter assay is considerably more complex. The objects of the research project were:

1. to determine if these two assays give the same information about donor-specific responsiveness/unresponsiveness;
2. to determine, in sequential studies, if serial

measurements of responsiveness may predict rejection, and if unresponsiveness could be used as a means of reducing immunosuppression to a safe minimum.

At present, there is no measure of adequate immunosuppression, only inadequate, the latter indicated by allograft rejection. If these two assays give essentially the same information about donor-specific responsiveness, the simpler one could be used in a clinical setting to determine if inadequate immunosuppression and imminent rejection can be detected, and also if possible, in nonresponsive patients, to reduce immunosuppressive therapy; a highly desirable goal in long term immunosuppressed patients.

As shown in the results and will be discussed further in this section, the CML assay appears to give more clinically relevant information, though the suppressor cell assay is useful to dissect the mechanisms involved in the production of a state of allograft tolerance.

The patient population chosen for this study consisted of fifteen patients with normal renal function at one year, and fifteen patients who had totally rejected their allografts within the year, and hence reflected two ends of the spectrum of the transplant population. The reason for selecting these patients is, if the tests were proven to be discriminatory between these two groups, they could be useful in an average transplant population. On the other hand, if these tests were not useful in these two groups, it would be unlikely that they would show much change in an unselected transplant population.

The experimental assays for both of the tests done in this project used the micromethod. Though the original standardization for the CML assay utilized the macromethod (Lightbody et al., 1972), the micromethod has been well characterized (Zarling et al., 1976) and, in certain hands, is even more sensitive than the macromethod (Hirschberg et al., 1977). For this project, the microtechnique offered several advantages over the more conventional macro or bulk technique. The cell washing and transfer steps are eliminated, these are time consuming processes which may also lose some of the cytotoxic cells under study. More importantly, considerably less cells are required, which is a significant consideration in post-transplant immunosuppressed patients.

A. CML Assay; Kinetics and Cell Ratios

A persistently reduced recipient CML toward donor target cells in patients with normally functioning grafts raises the question of whether this effect is due to accelerated kinetics of the response. Experiments to exclude this possibility tested recipient effector cells against the appropriate targets on days 3 and 6. As shown in Figure 1, the post-transplant recipient cells CML against donor targets was negative on day 3 with only a slight increase toward day 6. The same recipient cells against a third party control was also negative on day 3, but showed a tenfold increase on day 6. These experiments performed with six recipient samples both pre- and post-transplant suggested that accelerated kinetics could not

explain the specifically reduced cytotoxic response against the donor.

Varying effector:target ratios were assessed in the CML assay to decide on the optimum ratio to be used in subsequent assays as well as to see if a functional clonal deletion is responsible for the reduced CML response. The results in Table VIII show an increase in lysis with increasing effector:target ratios, with the post-transplant recipient to donor CML showing reduced cytotoxic response at all effector:target ratios tested. To achieve a thirty percent lysis, more than twice the number of post-transplant cells would be needed as compared to the pre-transplant cells. This would suggest that there is a depletion of functionally active cytotoxic precursors in the post-transplant sample directed against the donor. However, whether this functional depletion of donor-specific cytotoxic precursors is due to a clonal depletion, suppressor cells or even antiidiotypic antibodies is not clear.

The effector:target ratio used in subsequent studies was 50:1, since this ratio was convenient for use and gave adequate lysis. The responder to stimulator ratio used was 1:1 for similar reasons. Details of various responder:stimulator ratios used and the specific lysis have been extensively discussed by Hirschberg et al. (1977).

B. Suppressor Cells: Characteristics

Experiments were carried out to characterize the radiosensitivity, dose and kinetics of suppressor cells in the assay system (Tables IX, X and XI).

There is a certain amount of information available on the radiosensitivity of in vitro generated suppressor cells. In general these cells, if induced by autologous MLC or mitogen, are sensitive to radiation and mitomycin C (Smith et al., 1979) and (Goeken et al., 1981); if induced by allogeneic stimulation they are less sensitive to radiation (Ferguson et al., 1977). However, there is hardly any information on the radiosensitivity of in vivo generated suppressor cells in clinical transplantation. Some authors have used irradiated or mitomycin C treated cells as putative suppressors (Liburd et al., 1978) and (Pfeffer et al., 1981), whereas others have used cells without any pretreatment in the assay (Goulmy et al., 1981) and (Charpentier et al., 1982). The experiments done in this project suggest that the putative suppressor cells are radiosensitive. In all the experiments subsequently performed, 1000 rads were given to the suppressor cells to prevent proliferation and production of cytotoxic effoctor cells against the donor cells. Whether a greater degree of suppression would be obtained if nonirradiated cells were used remains to be clarified.

'Varying responder to suppressor ratios have been used by various authors. Charpentier et al. (1982) showed that ratios of 1:1 to 1:0.25 gave adequate suppression, but that reducing

the suppressor cells further, the effect was lost. Liburd and Thomas used a 1:0.25 ratio, whereas Goulmy and Pfeffer used a 1:1 ratio. The experiments done here gave equivalent amounts of suppression with 1:1 and 1:0.5 ratios, the latter being used in subsequent experiments.

Kinetic studies on the suppressor cell assay showed no evidence of accelerated kinetics to explain the observed decrease in lysis.

C. CML Responsiveness

Previous studies have indicated that most transplant patients with well functioning HLA mismatched kidney allografts, either from living related or cadaver donors have a strongly reduced cell-mediated cytotoxicity toward the donor. The results presented here (Figure 3) confirm these studies and in addition, show the donor-specific reduced cytotoxicity to develop as early as one month post-transplantation. The recipient effector cells, taken one month after transplant, after stimulation for 6 days with donor cells, showed an average cytotoxicity of only 20%, whereas pre-transplant recipient cells showed a 40% specific lysis. This effect was unlikely to be due to a general lack of cytotoxic precursors caused by immunosuppression, since the same post-transplant recipient sample could generate strong cytotoxicity against third-party stimulator cells. In addition, Goulmy et al. (1981) have shown that post-transplant recipient cells, when stimulated with a pool of lymphocytes containing from five to

twenty unrelated cells, cytotoxic effector cells were produced against the pool target. These recipient cells, however, failed to react with the respective donor target, indicating a selective inactivation of the cytotoxic precursor cells. A correlation between the degree of CML unresponsiveness and the length of time of the surviving allograft has been suggested (Thomas et al., 1979). Pfeffer et al. (1983) demonstrated that all but one of the samples taken after two years post-transplant showed <10% lysis. In this study too, there is the suggestion that the CML unresponsiveness increases with sampling time post-transplant. However, samples after one year post-transplant were not assessed to confirm this. It would appear that there is agreement from various centres, that serial donor-specific hyporesponsiveness correlates well with normal graft function and could signify graft acceptance. The next step would appear to be to see if the immunosuppression given to these patients could be gradually reduced; in fact there is a preliminary report that one centre used the emergence of donor-specific MLR hyporesponsiveness to tailor the immunosuppressive regimen by withdrawing cortocosteroids from sixteen recipients of living related donor kidneys (Kahan, 1985).

In contrast to MLC/CML responsiveness in patients with normally functioning grafts, there are hardly any serial studies of CML responsiveness in patients with poor function. Goulmy et al in 1981, testing two post-transplant samples per patient (though without pre-transplant samples) observed that

CML reactivity over 35% rarely occurs after the first few weeks of transplantation. In 9 out of 45 patients, it was found to be substantially higher, and in 5 of them this coincided with a reversible rejection crisis. Thomas et al (1981) studied the donor-specific CML responses pre- and post-transplant in 25 recipients. Pre-transplant, all the recipients responded with significant in vitro cytotoxic lymphocyte responses to the specific donor. Post-transplant, two distinct patterns of CTL reactivity were observed: in one group of 14 patients, the cytolytic activity against donor cells had dropped to a less than 25% of the pre-transplant value within six months, whereas the other 11 patients did not show a drop. In the first group of 14 patients only one graft was lost due to acute rejection, whereas over half of the grafts in the second group were lost to acute rejection and the difference was statistically significant. This suggested that the degree of post-transplant in vitro CTL reactivity could correlate with states of quiescence or acute rejection. In this study, this aspect was assessed a bit differently; patients who were known to have lost their grafts due to severe rejection within the first year post-transplant were assessed for donor-specific CML inducibility both pre-transplant and at one and three months post-transplant. The results show that, in this group, the CTL reactivity remained elevated at the one and three month post-transplant period. However, in correlating the renal function with the time of sampling, it was observed that the mean serum creatinines was 354 μmols/L at the first month

post-transplant, suggesting that there was probably ongoing rejection in most of these. In 4 patients who had serum creatinines below 250 μmols/L the mean post Tx specific lysis was 28%, compared to a pre Tx value of 38%. Hence in this study we did find a correlation between high levels of CTL reactivity and rejection, but it could not be stated definitively, due to the few patients studied, that a high CTL reactivity post Tx is predictive of an acute rejection.

D. Suppressor Cells and Transplant Tolerance

The issue of the role of suppressor cells and transplant tolerance is one that is fraught with controversy. In fact, an extreme view by Moller (1985) states that he questions the existence of suppressor T cells as a distinct T cell subpopulation different from cytotoxic T cells! The reasons he gives to support his view are: (a) suppressor and cytotoxic T cells cannot be distinguished by markers, (b) the gene I-J governing suppressor T cell function cannot be found at the DNA level in the position where it has been mapped by classical genetic methods, (c) T cell clones have been identified with a variety of properties which do not easily fit the concept of three distinct T cell subsets. In addition, he mentions that in some of the test systems used the results may be due to in vitro artifacts. In a series of experiments he has shown that the suppressive effect of Con A activated T cells was totally abolished by adding T cell growth factor (TCGF) suggesting that deprivation of TCGF was the mechanism by which they exerted

their effect. However, the concept of suppressor T cells has been generally accepted by most workers, and has been demonstrated to be operative in various models of transplantation tolerance. The reason for the controversy is the disparate results obtained by various investigators working with similar models.

In this study, using the methodology described earlier, we have demonstrated the presence of suppressor cells in both groups of patients, the patients with normal function showing it to a greater degree. However, the mean drop in the specific lysis that could be demonstrated was not very striking. In well functioning patients, from a control value of 58%, with the addition of putative suppressor cells, the specific lysis dropped to 49%. This was statistically significant with the paired T test ($p<0.01$), but drops of this degree are difficult to assess in individual patients.

In patients with poor function, in some of whom suppressor cells could be demonstrated, the mean specific lysis in the control value was 43.5% which dropped to 37.5% post-transplant ($p < 0.025$).

These results would suggest that suppressor cells do exist in transplant tolerance, but for an individual patient the demonstration of suppressor cells need not correlate with graft function. This is quite understandable since, as mentioned earlier, the balance between rejection and antirejection forces would decide whether an allograft is tolerated. This has been demonstrated by Dossetor et al. (1981) in that suppressor cell

activity could be shown in patients with acute and chronic rejection.

In the experiments mentioned earlier, peripheral blood lymphocytes were used as putative suppressors. It is quite plausible that a low concentration of in vivo formed suppressor cells in the peripheral blood is responsible for low degree of suppression detected. In limited experiments, it was assessed if by using subsets of lymphocytes the degree of suppression detected would increase. As shown in Table XII there was an increase in suppression when T cells and T suppressor/cytotoxic enriched populations were used, and this would appear to be a more sensitive method of detecting suppressor cells, though procedurally much more involved. An interesting finding was that suppression of the control culture also showed an increase when the suppressor/cytotoxic subset was used, suggesting that both donor-specific and nonspecific suppressor mechanisms are probably being detected. However, Charpentier et al (1983) have demonstrated that OKT8+ and Fc gamma+ post-transplant lymphocytes exert a suppressive effect specific to the donor-recipient pair on the proliferative response of the pre-transplant lymphocytes.

Using subsets of lymphocytes appears to be one method of detecting suppression. Another method would be to use cells from lymph nodes or the spleen instead of the peripheral blood. This approach would not be applicable in clinical transplantation but rather in animal models. Using cells aspirated directly from the allograft with the fine needle

technique and cloning the various cells obtained would appear to be an ideal method in the future.

Another aspect of the loss of CML reactivity that was explored in this study, though unsuccessfully, was whether antiidiotypic antibodies could be responsible for the depressed CML reactivity. The protocol involved trypsinization of the recipients' cells, both pre- and post-transplant and setting up CML induction studies against the specific donor; this would be compared to non-trypsinized samples (Kedar et al., 1974). The principle involved is, if the T cell receptors of the recipient are blocked by antiidiotypic antibodies, treatment with trypsin should strip off the receptor and the blocking antibodies. Fresh receptors should regenerate within eight to twelve hours, and if the CML induction post-trypsinization shows an increased lysis, it would be suggestive that antiidiotypic antibodies blocking the receptors, were responsible for the CML hyporesponsiveness.

Various doses of trypsin were tried on normal cells and patients' cells, using both the lymphocyte mediated cytotoxicity assay (LMC) as well as the CML induction assay. However, with an occasional exception, in general the trypsinized samples gave less degrees of lysis (results not shown). This suggested that trypsin, in this experimental protocol, had a non-specific depressant effect on the CML induction and hence this aspect was not explored any further.

Finally, from this study, it appears that the donor-specific CML hyporesponsiveness is most likely caused by

multiple mechanisms. Suppressor cells specific to the donor have been demonstrated to a greater degree in patients with normally functioning grafts, and this would suggest that they too are important in the production of the state of donor-specific hyporesponsiveness. The relative contribution of this mechanism, as well as other postulated mechanisms, such as antiidiotypic antibodies and clonal deletion, however, cannot be answered at this stage.

V. CONCLUSIONS

1. The conditions for optimal detection of in vivo suppressor cells using this technique are:
   1. a responder:stimulator:suppressor ratio of 1:1:0.5;
   2. 6 days of in vitro culture.
2. In vivo suppressor cells appear to be radiosensitive and belong to the T cell subset.
3. A small degree of donor-specific suppression is demonstrated in the group with normally functioning grafts as compared to controls.
4. Donor-specific CML hyporesponsiveness develops in allograft recipients with normal renal function and a smooth post-transplant course, and can be detected as early as one month post-transplant.
5. Allograft recipients who have lost their graft due to severe rejection retain CML reactivity against the donor at one month post-transplant.
6. There appears to be some correlation between high donor-specific CML reactivity and poor allograft function due to rejection.
7. A prospective study with a large number of unselected patients would be required, to prove if the donor-specific CML reactivity at one month could be predictive of ultimate allograft outcome.

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